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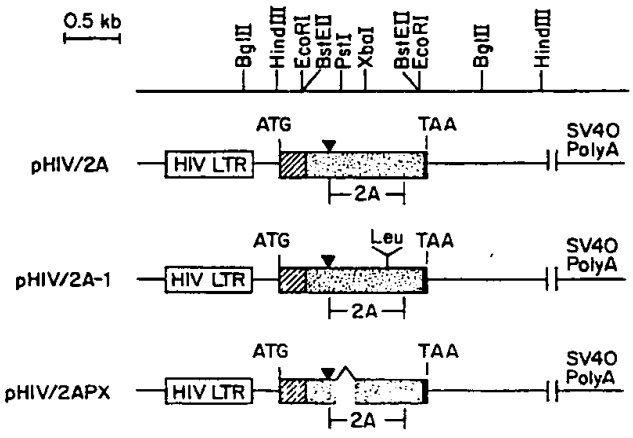

(57) Abstract

An intracellular method of inhibiting HIV in mammalian cells, in which a recombinant construct is introduced into the cells. The recombinant construct includes a) the HIV long terminal repeat (LTR) or a portion of the HIV LTR which includes a functional HIV promoter and DNA of non-HIV origin encoding a product which is toxic to HIV-infected cells, when present in such cells alone or in conjunction with a selected substance, or b) all of the HIV LTR or a portion of the HIV LTR which includes a functional HIV promoter and mutated or altered HIV DNA. Compositions for use in inhibiting HIV, which include such recombinant constructs, are also disclosed.

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INTRACELLULAR METHOD OF INHIBITING HIV IN MAMMALIAN CELLS

Description

Background

Human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS), which is characterized by immune suppression resulting from selective infection of T helper cells and death of OKT4+ T helper cells. Sarin, P.S. Ann. Rev. Pharmacol., 28:411-428 (1988). Clinical manifestations of the disease include severe immune deficiency, which is generally accompanied by malignancies and opportunistic infections.

There is a particular interest in finding ways to prevent infection by HIV and to counter the effects of the virus in an already infected individual because of the devastating effects of the virus and the fact that the mortality rate among such individuals is very high.

Despite the fact that much effort, time and money have been expended in developing a means of preventing HIV infection or of reducing or eliminating the effects of the virus once infection has occurred, only limited progress has been made to date in doing so. It has been suggested that various points in HIV infection of T cells and virus replication in infected cells should be targeted in developing chemotherapeutic agents useful in preventing or treating the disease. Such points in

the HIV life cycle as attachment to T cells, reverse transcriptase activity, DNA transcription and/or translation and assembly and release of virus particles might be effectively targeted. Several drugs, including those, such as D-penicillamine (DPA), which have been used in the past for treatment of other diseases, and those, such as anti-sense oligonucleotide inhibitors, designed specifically to interfere with HIV, have been assessed for their effectiveness in preventing HIV infection of cells and/or inhibiting the effects of the virus in infected cells. Sarin, P. Ann. Rev. Pharmacol., 28:411-428 (1988); Norman, C., Science, 30:1355-1358 (1985).

Although these efforts are, in some instances, producing promising results, it is clear that at the present time, there is no effective means for interfering with HIV activity in infected cells.

Summary of the Invention

The present invention relates to a method of reducing the effects of HIV in an HIV-infected individual by means of a recombinant nucleic acid sequence, or recombinant construct, expressed in HIV-infected cells, which encodes a product whose expression in such cells results in inhibition of the HIV, either directly or indirectly. The present invention also relates to the recombinant construct and a composition which includes the construct.

The recombinant nucleic acid construct comprises at least two components, one of which is at least a portion of the HIV genome. The portion

generally includes all or a segment of the HIV promoter (i.e., a sufficient segment for the promoter to be functional). Preferably, expression or activation of the HIV component of the recombinant construct occurs only in HIV-infected cells, is toxic only to HIV-infected cells, or both. The recombinant construct additionally comprises DNA of non-HIV origin or mutated or altered HIV DNA. The recombinant construct can be present in cells prior to their infection by HIV and/or can be introduced into cells after they have become infected with HIV.

In one embodiment of the present invention, a recombinant construct which comprises a portion of the HIV genome, or an equivalent nucleotide sequence, and DNA of non-HIV origin, is introduced into cells, in which it is expressed, resulting in production of a protein or polypeptide which acts to kill the HIV-infected cells. In this embodiment, the recombinant construct is referred to as an HIV/non-HIV construct. In this embodiment, the DNA of non-HIV origin can encode 1) a protein or polypeptide whose expression in sufficient quantity in HIV-infected cells causes death of such cells or 2) a protein or polypeptide whose expression in HIV-infected cells is toxic to (kills) such cells only in the presence of a second agent or substance. The second agent will generally be a drug selected for its ability to act in conjunction with the encoded protein or polypeptide to cause cell death. In either case, only HIV-infected cells are killed.

In a second embodiment of the present invention, a nucleotide sequence, referred to as a mutated or altered construct, which encodes a mutated HIV gag gene, or other mutated HIV gene encoding another protein provided in trans, is introduced into mammalian cells. Cells containing the mutated gag construct express HIV at lower levels than HIV-infected cells in which the mutated gag construct is not present. As a result, less virus is released from infected cells and further infection of cells is less than it would otherwise be.

The present invention provides a means by which the effects of HIV can be reduced (diminished or eliminated) through the activity of a gene product produced in HIV-infected cells. This intracellular production method makes it possible to kill HIV-infected cells selectively and to reduce or eliminate the production in HIV-infected cells of infectious virus.

Brief Description of the Drawings

Figure 1 is a schematic representation of the construction of the HIV/2A gene and its derivatives, with a restriction map of HIV/2A. The HIV-1 LTR is shown by the first box. The coding sequence of HIV/2A or its derivatives is shown by the second box, in which the CAT sequence, the poliovirus sequence and the random amino acids are represented by the hatched, shaded and black portions, respectively. The region in the polioviral sequence encoding 2A protein is bracketed underneath. The

junction of P1 and 2A is marked by an inverted triangle. The remainder of the sequence is represented by a thin line and the SV40 polyadenylation site is indicated.

Figure 2 shows the results of *in vitro* translation of the 2A and 2A-mutant fusion proteins. Panel a shows total translation products and panel b shows the results of immunoprecipitation. RNA's used for translation contain the following coding sequences: Lane 1, mock; lane 2, CAT; lane 3, 2A; lane 4, *2A-1*; and lane 5, 2APX.

Figure 3 demonstrates the inhibitory effect of 2A protein on mRNA translation. Panel a shows results of experiments in which HeLa cells were cotransfected with equal molar amounts of pRSVCAT, pSVETA or carrier DNA, as indicated, plus one of the following: carrier DNA (lanes 1 and 2); pHIV/2A (lanes 3 and 4); pHIV/*2A-1* (lanes 5 and 6); pHIV/*2APX* (lanes 7 and 8). CAT activities were measured and expressed as the percentage of conversion from chloramphenicol to acetylated chloramphenicol. Panel b shows results of experiments in which the HeLa 2A-38 cell line was cotransfected with 10 ug of pRSVCAT, with or without 10 ug pSVETA, and CAT activities were determined. Panel c is a representation of the rationale of the experimental design.

Figure 4 demonstrates the cleavage of P220 in 2A-producing HeLa cells. The immunoblot experiment was performed using an antiserum against P220, essentially as described by Bernstein *et al.*, except that an alkaline phosphatase conjugated anti-rabbit

IgG (Promega) was used as the second antibody according to the vendor's instructions. Extracts were prepared 48 hours post-transfection from: lanes 1 and 2, HeLa cells transfected with or without pSVETA; lanes 3 and 4, 2A-38 cells transfected with or without pSVETA. Extracts were also prepared from HeLa cells infected with wildtype (lane 6) or 2A-1 mutant virus (lane 5) at multiplicity of infection of 20 for 3.5 hours. One fifth of the extracts from each 60 mm dish was analyzed by electrophoresis through a 6.5% SDS-polyacrylamide gel. P220 and its cleavage products are indicated.

Figure 5 is a schematic representation of recombinant retroviral constructs comprising a portion of the HIV promoter and a gene of non-HIV origin (here, the Herpes simplex virus thymidine kinase (TK) gene).

Figure 6 is a photograph of Southern blots showing the correct structure of integrated proviral DNAs in infected 143 osteosarcoma cells or 143 osteosarcoma cells containing the tat gene sequence.

Figure 7 is a photograph showing effects of acyclovir (ACV) at two concentrations on recombinant HIV-TK retrovirally infected 143 tat cells (left, 0μM ACV; middle 10μM ACV; right, 100μM ACV).

Figure 8 is a schematic representation of mutated constructs used to transfect mammalian cells.

Figure 9 is a schematic representation of the genomic organization of HIV-1 (top panel) and of various gag and rev mutants. In the top panel, the

gag region is enlarged underneath, with nucleotide numbers indicating the initiation codon, the cleavage sites between p17, p24 and p15, and the gag termination codon and in the remaining panels (I-VII) the various mutants are represented. I and II contain a stop codon in the p17 and p24 sequence, respectively. III and IV have a three- and four-amino acid insertion in the p24 protein, respectively. V has a four amino-acid insertion in the Rev coding sequence, as well as a stop codon in the tat gene. VI and VII have an in-frame deletion in the gag precursor.

Figure 10 is a schematic representation of constructs used to create HT4(ΔE-dhfr) cell lines. The broken arrow refers to the env translational frameshift, the thick line upstream of the 3' LTR to the sequence coding for a mutant dihydrofolate reductase. The dotted line in I-ΔE-dhfr indicates the presence of a stop codon in the p17 coding sequence, the dark triangles in III-ΔE-dhfr and V-dhfr represent linker insertions in the p24 and Rev coding sequences, respectively, and the broken line in VI-ΔE-dhfr corresponds to the deletion described in Figure 9.

Figure 11 shows results of immunoblot analysis of cytoplasmic extracts from HT4(ΔE-dhfr) cells. Lanes: 1, HT4-6C (negative control); 2, HT4(R7-dhfr) (infected with a replication competent version of HIV); 3, HT4(WT-ΔE-dhfr); 4, HT4(III-ΔE-dhfr); 5, HT4(VI-ΔE-dhfr).

Figure 12 is a graphic representation of p24 activity in the supernatant from HIV-infected HT4(ΔE-dhfr) cells.

Figure 13 is a graphic representation of virus yield produced by HIV-infected HT4(ΔE-dhfr) cells.

Detailed Description of the Invention

The present invention is based on the discovery that the adverse effects of HIV on mammalian cells infected with the virus can be reduced (inhibited or eliminated) by expression in such cells of a nucleotide sequence which includes at least a portion of the HIV genome. The portion of the HIV genome in the cells can be present in a recombinant construct which also includes DNA of non-HIV origin or altered or mutated HIV DNA. The portion of the HIV genome present in the recombinant construct includes all or a portion of the HIV long terminal repeat (LTR). The LTR or portion thereof present in such a recombinant construct includes, in turn, all or a portion of the HIV promotor; if only a portion of the HIV promoter is present in the construct, it is a sufficient segment for the promoter to be functional. The recombinant construct additionally comprises 1) DNA of non-HIV origin which encodes a product whose expression in HIV-infected cells is toxic to (causes death of) the cells or 2) altered or mutated HIV DNA whose expression reduces (inhibits or eliminates) the production of infectious virus by HIV-infected cells. These two components are present in recombinant constructs of this invention, in such a relationship to one

another that the HIV LTR or LTR portion controls expression or activation of the non-HIV DNA or of the altered or mutated HIV DNA.

In the case in which the recombinant construct includes DNA of non-HIV origin, the encoded product produced when the DNA is expressed may itself be toxic to cells, or the product may require the presence of another substance to be toxic to cells. In either of these cases, HIV-infected cells are killed selectively, as described in greater detail below, and non-HIV infected cells are not significantly affected. In the case in which the recombinant construct includes all or a portion of the HIV genome containing a mutation or an alteration, expression of the construct in cells reduces (inhibits or eliminates) the production of infectious HIV, thus reducing further infection of other cells.

The following is a description of various embodiments of the present intracellular method of inhibiting HIV in mammalian cells.

Introduction of a Recombinant Construct Comprising a Portion of the HIV Genome and a Gene of Non-HIV Origin

In one embodiment of the present invention, a construct comprising a portion of the HIV genome and all or a portion of a gene of non-HIV origin which encodes a product capable of interfering with HIV function in infected cells is introduced into cells. This recombinant construct is referred to as an HIV/non-HIV construct. It can include the HIV long

terminal repeat (LTR) or a portion thereof and DNA encoding a protein or polypeptide which is toxic to cells when expressed in sufficient quantities. For example, as described in Example 1, such an HIV/non-HIV recombinant construct can include the HIV LTR and DNA encoding all or a portion of the poliovirus protein 2A; the HIV LTR serves as the controlling element for expression of protein 2A. This construct is introduced into mammalian cells using known techniques. In HIV-infected cells in which the transactivator (tat) gene is present, the LTR is activated and the poliovirus protein 2A is produced, with the result that protein synthesis in the cells is blocked and cell death occurs. In this embodiment, although the HIV LTR/poliovirus protein 2A gene construct can be present in HIV-infected cells and HIV-free cells, it will be activated only in HIV-infected cells (because of the role of tat in its activation). Thus, it provides a means for selectively killing HIV-infected cells.

As described in Example 1, such a construct has been made and shown to be effective in inhibiting cellular mRNA translation. In this instance, a construct (designated pHIV/2A) was produced to express the poliovirus 2A protein in mammalian cells. As shown in Figure 1, pHIV/2A includes a 695 BstEII fragment of poliovirus (PV) cDNA (Mahoney strain), which is in frame with the coding sequence of a bacterial chloramphenicol acetyl transferase (CAT) gene, whose expression is under the control of the HIV-1 LTR. Specifically, the coding sequence of the fusion gene includes: 73 N-terminal amino acids

of CAT; a poliovirus sequence including 50 C-terminal amino acids, the entire 2A protein region and 33 N-terminal amino acids of protein 2B, and nine random amino acids which precede a stop codon. Other fusion genes, which contain mutations in the 2A-encoding sequence, were also constructed, as described in Example 1. One fusion gene, designated pHIV/2A-1, contains a single amino acid insertion in 2A, which results in a mutant poliovirus (2A-1) defective in shutting off host protein synthesis upon infection. The other fusion gene, designated pHIV/2APX, has a 53 amino acid deletion in 2A.

As results described in Example 1 and represented in Figure 3 demonstrate, in mammalian cells co-transfected as described, the expression of tat resulted in greatly reduced synthesis of CAT when cells were co-transfected with a construct containing the CAT gene (pRSVCAT) and the pHIV/2A construct which expresses poliovirus protein 2A (compared with CAT expression in cells containing no pHIV/2A). Without pHIV/2A, no inhibition of CAT synthesis was evident. Results of similar cotransfection experiments using a pRSVLACZ plasmid or a pSV2CAT plasmid as the reporter gene were equivalent to those described in Example 1, suggesting that inhibition of translation by protein 2A is not specific to a particular protein, promoter or mRNA capping sequence.

In this embodiment, a recombinant construct, such as pHIV/2A, in which the HIV LTR controls the expression of a protein (e.g, the poliovirus protein 2A), which inhibits (reduces or eliminates)

translation of cellular mRNA when HIV is present, is introduced into cells as a means of countering the effects of HIV. Such a recombinant construct can be introduced into cells prior to infection by HIV (e.g., in anticipation of exposure to/infection by HIV) or after infection has occurred. Only upon activation of the HIV LTR, which will occur in cells containing HIV, will expression of the nucleotide sequence of the recombinant construct occur, resulting in production of poliovirus 2A. Poliovirus 2A will block mRNA translation (protein synthesis) only in HIV-infected cells and, thus, will be toxic to (cause cell death) HIV-infected cells only.

Alternatively, an HIV/non-HIV construct can include all or a portion of the HIV promoter and DNA encoding a product, which alone is not toxic to cells in which it is expressed, but in the presence of a selected substance (i.e., in conjunction with a selected substance or agent) is toxic to cells. The non-HIV DNA encoding such a product is present in the recombinant construct in such a manner that its expression is controlled by the HIV promoter or promoter portion. For example, as described in Example 2, selective ablation of HIV-infected cells can be effected through expression of a product, such as thymidine kinase (TK) encoded by the Herpes simplex virus gene, in cells in the presence of a substance, such as acyclovir. In this embodiment, expression of the Herpes simplex TK gene is under the control of a minimal promoter element of the HIV LTR.

In this instance, a recombinant construct comprising the minimal promoter and the TK gene is introduced into cells by known techniques (e.g., by transfection, by means of a retrovirus or other suitable vector). Once present in the cell, the HIV promoter will function only in the presence of transactivator protein (tat), which is present only in HIV-infected cells. When the HIV LTR promoter element is activated by tat, the TK gene is expressed. Thus, in HIV negative cells (in which tat is not present), the HIV LTR promoter is inactive and no TK will be expressed. In HIV-infected cells, however, in which tat is present, the HIV LTR promoter is functional and TK is expressed. By itself, TK does not have adverse effects on cells. However, in the presence of acyclovir or analogues thereof (e.g., Gancyclovir, FIAU), TK is toxic to cells. Thus, only HIV-infected cells will be eliminated by the combined actions of TK and acyclovir. In this instance the construct component of non-HIV origin is the TK gene and the additional substance necessary to cause the desired adverse effect on HIV-infected cells is acyclovir. However, it is also possible to include in the recombinant construct any gene or gene portion encoding a product which has the same characteristics (i.e., lack of toxicity or adverse effect in the absence of a selected substance and ability to ablate HIV-infected in the presence of the selected substance).

The recombinant construct can be introduced, for example, into haematopoietic stem cells, which are the primary target of HIV infection. Blood cells, T-lymphocytes and monocytes/macrophages derive from haematopoietic stem cells, which can be purified prior to introduction of the construct. For example, bone marrow cells, which contain haematopoietic stem cells, can be taken from an individual, for example, one thought to be HIV-infected. The total (mixed) cell population or a purified portion of the stem cells can be infected or transfected with the recombinant retroviral construct, or can be introduced by other means, such as transfection. The bone marrow stem cells containing the construct can be introduced (injected) into the individual. To facilitate growth of the introduced, construct-containing cells, the individual's bone marrow can be partially cleared by irradiation or through use of a cytotoxic drug.

into the individual to provide a more direct route. In this case, preferably the retrovirus will have or be modified to include a surface protein that is stem-cell tropic, thus making it possible for it to be targeted to marrow cells.

Introduction of an Altered or Mutated Recombinant Construct Expressing an Altered or Mutated HIV Gag Protein

In a second embodiment of the present invention, a construct comprising mutated or altered HIV DNA encoding a mutated or altered HIV protein

provided in trans is introduced into cells, prior to or following infection of the cells by HIV. As used herein, mutated or altered refers to a sequence (nucleic acid sequence or amino acid sequence) which differs from the corresponding HIV sequence (nucleic acid sequence) or HIV-encoded sequence (amino acid sequence) by one or more changes (additions, deletions) in its constituents (i.e., nucleic acid(s), amino acid(s)).

In one embodiment, the mutated or altered HIV DNA is DNA encoding a mutated or altered HIV gag protein. Expression of the mutated construct results in reduction of the quantity of HIV released from infected cells and, thus, a reduction in HIV available to infect additional cells.

It has been shown that HIV gag mutants can interfere with the adverse effects of HIV on infected mammalian cells by conferring a dominant negative phenotype on cells in which the mutants are expressed. When a selected gag mutant is expressed, its product dominantly interferes with the normal function of the product of the parent, or wild type, gene. It has been shown that such gag mutants interfere with the generation of infectious viral particles from cells in which they are cotransfected with a wild type proviral DNA. It has also been shown that cells which constitutively express such HIV gag mutants have an impaired ability to support HIV replication when infected with wild type virus.

By means of the present method, it is possible to introduce into an individual, prior to, at the time of or after infection by HIV, a recombinant

construct whose expression confers a dominant negative phenotype on the cells into which it is introduced (e.g., hemato-poietic stem cells). The construct includes a selected HIV gag mutant and a functional HIV promoter, under the control of which the gag mutant is expressed.

The following is a discussion of dominant negative mutants and a description of this embodiment of the present intracellular method of inhibiting HIV in mammalian cells, the construct useful in the method and cell lines which constitutively express altered or mutant HIV gag sequences and in which generation of infectious HIV particles is inhibited as a result of the activity of the gag mutants.

Dominant Negative Mutants

Mutations can confer a dominant negative phenotype on cells: when a gene carrying such a mutation is expressed, its product can dominantly interfere with the function normally accomplished by the product of the parental gene. Herskowitz, I., Nature, 329:219-222 (1987). When the protein is multimeric, an effective dominant negative variant can be one making a monomer that is still capable of interacting with the wild type polypeptide chains but is otherwise defective, and that can recruit wild type monomers into non-functional multimers. In such a case, only a moderate level of expression of the dominant negative mutant might be sufficient to exert a strong inhibitory effect on the parental protein.

The generation of dominant negative mutations, which has been called intracellular immunization, (Baltimore, D., Nature, 335:395-396 (1988)), has been shown to be a potential strategy for controlling human immunodeficiency virus (HIV) infection, because the spread of the virus in an individual results mostly, if not solely, from infection of cells derived from a single precursor, the hematopoietic stem cell. If this precursor can be modified in such a way that its progeny does not support HIV replication, and if a patient's hematopoietic elements can be reconstituted from cells all derived from such a precursor, then the spread of the virus might be prevented in this patient. Such a scheme poses the same problems as any gene therapy procedure, but a preliminary challenge is to identify effective dominant negative variants of HIV genes. There are at least three distinct HIV genes which are potential targets for the generation of dominant negative mutants: tat, rev and gag. Because of the highly multimerized state of the gag products in the mature virion, dominant negative mutants of gag may be the most effective.

Like other retroviruses, HIV synthesizes its major internal structural proteins as a polypeptide precursor ($Pr55^{gag}$). This precursor is both phosphorylated and myristylated, and is then cleaved by the virally-encoded protease to yield the mature viral proteins, p17, p24 and p15; the latter is believed to exist as p9 and p7 polypeptide species. Veronese, F.d.M., et al, Virol., 62:795-801 (1988):

Mervis, R.J., et al, Virol., 62:3993-4002 (1988). By analogy with other retroviruses, HIV gag proteins are likely to play a role in viral assembly and release, in stabilization of the virion, in uncoating of the viral RNA, in the initiation of reverse transcription, and perhaps in integration. Crawford, S. and S.P. Goff, J. Virol., 49:909-917 (1984); Schwartzenberg, P., et al, J. Virol., 49:918-924 (1984); Hsu, H.W., et al, Virology, 142:211-214 (1985); Meric, C. and Spahr, P.-F., J. Virol., 60:450-459 (1986); Prats, A.C., et al., EMBO J., 7:1777-1783 (1938); Meric, C. and S. Goff, J. Virol., 63:1558-1568 (1989); Schultz, A.M. and A. Rein, J. Virol., 63:2370-2373 (1989); Bowerman, B., et al, Genes and Devel., 3:469-478 (1989).

As described below, it has now been shown that a number of HIV gag mutants can interfere with the generation of infectious viral particles from cells in which they were co-transfected with a wild type proviral DNA. In addition, it has also been found that cells constitutively expressing such HIV gag variants have a severely impaired ability to support HIV replication when infected with wild type virus.

As described in Example 3, mutated constructs containing a mutated gag gene and expressing the defective protein, were individually cotransfected into mammalian cells with wild type (WT) HIV, using known techniques. A control construct, in which the HIV genome contains a frameshift in the env region, was contransfected into mammalian cells under the same conditions, as a means of verifying that results obtained were due to a change in the gag

sequence. Constructs are represented schematically in Figure 8. RipAn has a HIV 5' LTR and no 3' LTR; a pro-insulin polyadenylation sequence is present at the 3' end. The sequence between the 5' LTR and 3' polyadenylation sequence is the HIV sequence. RipAmen has a 5' LTR, a polyadenylation site in place of the 3' LTR and a frameshift in the env region. Thus, it does not express HIV envelope protein. Mutated constructs designated by CH, followed by a number, have a linker insertion in the HIV sequence at the approximate position indicated by the number. That is, CH10 has a linker insertion at position 1000, CH14 at position 1400, etc. Mutated constructs whose designations begin with Δ, include a deletion between two sites indicated by the remainder of the designation (e.g., for ΔH10X12, a deletion between approximately positions 1000 and 1200). Constructs designated R7. (H10X12, P14S15, H10H17) are the corresponding HIV (i.e., HIV in which the corresponding deletion is present), with a viral 3' LTR.

Cells cotransfected in such a manner were cultured under appropriate conditions and supernatant from the cells was subsequently used to infect H9 cells. Infected H9 cells were cultured, harvested periodically and assayed, using anti HIV anti-serum and fluorescently labeled antibodies, using known techniques.

Results are presented in Tables 1 and 2 (Example 3) and demonstrate that cells infected with the WT HIV and gag mutant constructs produced less HIV (less HIV was released into the culture media)

than cells infected with WT HIV alone or with WT HIV and the control construct.

The ability of various gag variants, constructed as described in Example 4, to interfere with the replication of wild type virus was assessed, as described in Example 5, in a transient assay and as described in Example 6, in cells which constitutively express the HIV gag variants.

In the transient assay, COS cells were co-transfected with the intact wild type proviral DNA (W13) and each of the constructs (See Table 4). As described in Example 4, the supernatant was used to infect H9 cells. Immunofluorescence assay of the infected H9 cells with serum from an HIV-seropositive individual was used to evaluate the release of infectious particles from the co-transfected COS cells. Results showed that co-transfection with W13 (intact wild type proviral HIV DNA) and III-ΔE, IV-ΔE, VI-ΔE or VII-ΔE appeared to reduce dramatically the infectious yield from the COS cells. Thus, these gag variants had a major inhibitory effect on generation of infectious particles from the wild type provirus. These gag variants are dominant negative HIV mutants.

For the first time, a stable cell line has been constructed which is refractory to HIV replication by virtue of its expression of an altered HIV gene product. Cells which constitutively express the HIV gag variants were created and assayed for their ability to support HIV replication, as described in Example 6. Results demonstrated that H9 cells innoculated with the supernatant from two cells

(HT4(III-ΔE-dhfr; HT4(VI-ΔE-dhfr)) constitutively expressing gag mutants released very few infectious particles. They also showed that supernatants of two cells (HT4(I-ΔE-dhfr); HT4(III-ΔE-dhfr)), which contained grossly comparable amounts of p24 activity, were very different in terms of infectivity. That is, the supernatant from HT4(I-ΔE-dhfr) is highly infectious and that from HT4(III-ΔE-dhfr) appears to contain mostly non-infectious particles (see Figure 13). The impaired ability to support HIV replication in HT4(III-ΔE-dhfr) and HT4(VI-ΔE-dhfr) was also shown not to be due to the loss of CD4 receptor during cloning. Further, the block generated by the gag variants was shown to affect the late stages of the virus cycle.

Using the method described in Examples 3-6 and a mutated construct encoding a gag mutant (e.g., pΔH10X12, pΔP14A20, pΔ10H17), it is possible to reduce production in and, thus, the quantity of HIV available for release by infected mammalian cells. This can be carried out in an uninfected individual (e.g., an uninfected individual at high risk of HIV infection) or in an infected individual, using techniques described above for a recombinant construct comprising a portion of the HIV genome and a gene of non-HIV origin. Alternatively, a mutated construct encoding a gag mutant can be introduced into peripheral blood cells removed from the body and returned to the individual after introduction of the construct.

Although gag proteins have been targeted in the constructs described, it is also possible to use constructs containing an appropriate mutation in a gene encoding any protein which, like the gag-encoded protein, is provided in trans. This approach, which is a method of inactivating genes by a dominant negative mutation, makes use of an altered gene encoding a mutant product which, when expressed, inhibits the wild-type (HIV) gene in a cell, with the result that the gene product is non-functional. The result in the present invention is the reduction (inhibition or elimination) in production of infectious HIV.

Cell lines which include one of the following genes, introduced into the HIV genome at NEF, were made: the neo gene, which encodes G418 resistance; the dhfr gene, which encodes resistance to methotrexate; the hygro gene, which encodes resistance to hygromycin B; the gene encoding β-galactosidase; and the neo gene with internal regulatory sequences (promoter, polyadenylation signal). In addition, πAN7, which is a bacterial plasmid carrying an origin of replication and a suppressor tRNA gene to complement amber mutations in either a λ phage, a bacteria or a bacterial plasmid, was introduced into cells, which then constitutively express the encoded product. Because they contain a marker-encoding gene incorporated within the genome, all cells which express the marker-encoding gene also express HIV and, thus, can be identified by the presence of the encoded substance. Such cells can be used, for example, to

titer HIV; to select for viruses which differ from the parental virus; and to carry out HIV expression at high levels in target cells (e.g., in cells used to produce an HIV vaccine).

It has now been shown that HIV gag mutants can exert a powerful inhibitory effect on the replication of the wild type virus in susceptible cells. When cells constitutively expressing such HIV gag variants are infected with wild type virus, their infectious yield is dramatically reduced, compared to the yield of cells expressing wild type or no gag proteins. However, mutant gag-producing cell lines fully support all steps of viral replication that precede and result in viral gene expression, showing that interference occurs during the late stages of the viral life cycle, at the level of or following assembly.

It is likely that upon infection of mutant gag cell lines with wild type HIV, chimeric gag multimers are formed, containing a mixture of wild type and mutant gag monomers. The resulting structures are unable to carry out the steps that normally permit the transfer of the viral genome from one cell to another. The block may reside at the level of viral assembly or release, of virion stabilization, or of viral entry or uncoating. The degree to which those different steps are affected may vary depending on the nature of the gag mutant: VI, for instance, which has a sixty two amino-acid deletion overlapping the p17/p24 junction, seems to interfere mainly with viral assembly and/or release, because little p24 activity appears in the

supernatant of cells expressing this variant whether or not they are infected with wild type HIV. By contrast, III, which carries a four amino acid-insertion in p24, appears to allow the production of some viral particles, but these are mostly non infectious (Figures 12 and 13).

One of the gag variants tested produced a protein with an extensive deletion in the gag precursor, and removed the p17/p24 cleavage site (VI). Still, it exhibited a major dominant negative effect. This suggests that multimerization of the gag monomers must occur before processing, and that grossly modified precursors can still interact with the wild type polypeptide chains.

Because they target a highly multimerized HIV protein, relatively low levels of expression of the mutant allele may be sufficient to repress wild type virus replication. That may constitute a decisive advantage over dominant negative mutants in the tat or rev genes, which are not likely to be as efficient. Cells containing dominant negative gag mutants may still die following infection with wild type HIV; but if all blood cells in a patient could be derived from a precursor expressing such mutants, and therefore be unable to produce infectious particles, the viral spread in the patient would be limited, whatever the fate of the infected cells. Given the difficulties encountered in developing an anti-HIV vaccine and the problems of designing effective therapeutic agents, the present method and constructs have considerable potential as a means of anti-HIV intracellular immunization.

Using the method described in Example 3 and a mutated construct encoding a gag mutant (e.g., pΔH10X12, pΔP14A20, pΔ10H17), it is possible to reduce production in and, thus, the quantity of HIV available for release by, infected mammalian cells. This can be carried out in an uninfected individual (e.g., an uninfected individual at high risk of HIV infection) or in an infected individual as follows: The recombinant construct can be introduced, for example, into haematopoietic stem cells, which are the primary target of HIV infection. Blood cells, T-lymphocytes and monocytes/macrophages derive from haematopoietic stem cells, which can be purified prior to introduction of the construct. For example, bone marrow cells, which contain haematopoietic stem cells, can be taken from an individual, such as, one thought to be HIV-infected. The total (mixed) cell population or a purified portion of the stem cells can be infected or transfected with the recombinant retroviral construct, or can be introduced by other means. The bone marrow stem cells containing the construct can be introduced (injected) into the individual. To facilitate growth of the introduced, construct-containing cells, the individual's bone marrow can be partially cleared by irradiation or through use of a cytotoxic drug. Alternatively, the construct-containing composition can be injected into or otherwise administered to the individual to provide a more direct route. In this case, the retrovirus will preferably have or be modified to include a surface protein that is stem-cell tropic, thus

making it possible for it to be targeted to marrow cells. Alternatively, a mutated construct encoding a gag mutant can be introduced into peripheral blood cells which have been removed from the body and are returned to the individual after introduction of the construct.

Another potential therapeutic implication is illustrated by the analysis of mutant VI, which contains a deletion encompassing the cleavage site between p17/p24. This mutant exhibits a strong trans-acting inhibitory effect on wild type HIV-1 replication. Based on this logic, even partially effective HIV protease inhibitors might be of tremendous value in limiting viral spread, if they could induce in infected cells the production of a certain amount of uncleaved gag precursor. Those uncleaved precursors would probably interact with the wild type gag monomers, resulting in the formation of potentially non-functional multimers. As a consequence, production of infectious viral particles by infected cells might be dramatically reduced.

Sources of Components of the Recombinant Constructs of the Present Invention

In each of the embodiments described herein, the recombinant construct (i.e., HIV/non-HIV; altered or mutated) used can be constructed using DNA obtained from sources in which they occur naturally or produced by known techniques (e.g., mechanical or chemical methods). That is, HIV sequences present in the recombinant constructs can

be obtained from the HIV or can be DNA having the same nucleic acid sequence as the selected region(s) of the HIV genome which has been obtained using known cloning techniques, by chemical or mechanical synthesis, etc. It is not necessary that the DNA sequence be precisely the same as that of the selected region of the HIV DNA. Rather, it is possible to use DNA which is the functional equivalent of the HIV sequence (i.e., a sequence which encodes a product having the same amino acid sequence or function as that encoded by the naturally-occurring DNA). This is also the case for non-HIV DNA present in the recombinant constructs (e.g., Herpes simplex virus TK gene, poliovirus protein 2A-encoding DNA). It can be all or a portion of a selected gene and can be obtained from sources in which they occur in nature or can be synthesized using known cloning, mechanical or chemical methods. They can have the same sequence as that of the selected gene or a sequence which is its functional equivalent.

Recombinant constructs of the present invention can be administered to an individual in a pharmaceutical or therapeutic composition whose components are selected according to the method by which it is to be administered. Such compositions can include, for example, a suitable buffer, carrier and/ or adjuvant.

The present invention is further illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLE 1 Construction and Expression of HIV/2A

The human immunodeficiency virus (HIV-1) long terminal repeat (LTR) has been used as a controlling element for expression of polio virus protein 2A in HeLa cells, as described in this example. Results show that 2A by itself dramatically inhibits cellular mRNA translation. Cleavage of P220 following 2A expression was also detected. It has also been shown that the LTR of HIV-1 and its trans-activator (tat protein) act as a powerful, controlled expression system.

In order to express the poliovirus protein in cells, the following steps were carried out: A plasmid, designated pHIV/2A, was constructed by fusing a 695-bp BstEII fragment from a poliovirus cDNA (Mahoney strain) in frame with the coding sequence of a bacterial chloramphenical acetyltransferase (CAT) gene, whose expression is under the control of the HIV-1 LTR (Figure 1). Plasmid pHIV/2A was deposited (March 1, 1989) under the terms of the Budapest Treaty in the American Type Culture Collection (Rockville, MD) under ATCC #40578. The plasmid will be unconditionally and irrevocably released to the public upon issuance of a U.S. patent to Applicants.

To generate pHIV/2A, a BstEII fragment (3235-3930) from a poliovirus cDNA was filled-in with DNA polymerase (Klenow fragment), attached to a 12-mer EcoRI linker, and then inserted into the EcoRI site of the CAT gene driven by HIV-1 LTR in a plasmid, called 933A (Sodroski et al., W. Science 227: 171-173 (1985). This fragment is in frame with

the CAT gene at its N-terminus, but out of frame at its C-terminus, which creates a stop codon following 9 random amino acids. In the transcription unit of pHIV/2A, also residing between the stop codon and the SV40 polyadenylation site are a 3' portion of the CAT gene, 900 bp of truncated envelope sequence of HIV-1 and the intervening sequence of SV40 (not indicated in Figure 1).

pHIV/2A-1 was constructed by replacing the BstEII fragment in pHIV/2A with the BstEII fragment from the 2A-1 mutant cDNA. To generate the deletion in 2A of pHIV/2APX, HIV/2A plasmid was digested with Xbal (nucleotide 3581 in poliovirus sequence), and filled-in the XbaI site. This linear DNA was then partially digested with PstI, and the fragment that was only digested at the PstI site in the poliovirus sequence (nucleotide 3420) was selected using a 1% low-melting point agarose gel. The two ends of this fragment were ligated with a polylinker fragment with a PstI end on one side and a blunt end on the other, isolated from the Bluescript plasmid (Strategene) by digesting with PstI and EcoRV.

Thus, the coding sequence of the fusion gene contains 73 N-terminal amino acids of CAT; the polioviral sequence including 50 C-terminal amino acids of region P1, the entire 2A and 33 N-terminal amino acids of protein 2B; and 9 random amino acids preceding a stop codon. Similar fusion genes, designated pHIV/2A-1 and pHIV/2APX respectively, were also constructed with mutations in the 2A sequence (Figure 1). pHIV/2A-1 contains a single amino acid insertion in 2A, which results in a

mutant poliovirus, 2A-1, which is defective in shutting off host cell protein synthesis upon infection. pHIV/2APX has a 53-amino acid deletion in 2A.

Protein 2A has a proteolytic activity and is known to cleave the junction between P1 and itself. Thus, it was expected that the predicted 41 kd (kilodalton) fusion protein from the constructs would be processed by 2A, resulting in a mature protein of 24.5 kd containing only 2A and a small part of 2B. That such processing occurred was demonstrated by examining the in vitro-translated products from the coding sequences of the fusion proteins in various constructs.

To synthesize RNA's in vitro, the HindIII-BgIII fragments (Figure 1) containing the coding sequences of the fusion proteins were excised from HIV/2A, 2A-1 and 2APX, and ligated to the HindIII and BamHI sites of SP64. A HindIII-BgIII fragment containing the CAT reading frame was also cloned into SP64 to synthesize a control template. The coding strands were synthesized with SP6 polymerase (Promega). Approximately 1mg of RNA was used for the in vitro translation in 50 ul of the nuclease-treated rabbit reticulocyte lysate (Promega) containing ^{35}S-methionine according to the vendor. Five ul of each reaction mix was diluted in 1 ml immuno-precipitation buffer and centrifuged at 40,000 rpm in an SW50.1 rotor (Beckman) for 30 min. Ten ul of each was loaded on a 20% SDS-polyacrylamide gel for a. The remainder was immunoprecipitated with an anti-2A serum and half of that was loaded for b.

Figure 2 shows the results of in vitro translation of the 2A and 2A-mutant fusion proteins. Panel a shows total translation products and panel b shows the results of immunoprecipitation. RNA's used for translation contain the following coding sequences: Lane 1. mock; lane 2, CAT; lane 3, 2A; lane 4, 2A-1; and lane 5, 2APX.

The RNA containing the coding sequence of pHIV/2A directed synthesis of a 24.5 kd protein (Figure 2A, lane 3) that was immunoprecipitated by anti-2A serum (Figure 2B, lane 3). The coding sequence should direct synthesis of a 41 kd protein but processing is apparently so complete that none remains; the 17 kd P1 cleaved polypeptide was not detected and may have been degraded. The partially defective mutant 2A-1 gave rise to 24.5 kd and 41 kd products, both of which reacted with anti-2A serum (Figure 2, lane 4), implying that mutant 2A-1 is defective in cleavage of 2A from its precursor and confirming that a 41 kd product is made from the construct. In pHIV/2APX, the 53 amino acid deletion caused synthesis of a major 33 kd immunoprecipitable product (Figure 2, lane 5). The minor 27 kd product is presumed to result from alternative initiation from an inframe AUG or could be an abberant non-specific cleavage product. Specific cleavage at the P1/2A junction would have generated a much smaller product. Thus, the constructs produced the predicted products, implying that 2A is proteolytically active and that the mutants partially or completely block specific cleavage.

The ability of 2A to inhibit cellular mRNA translation was also examined, through the use of cotransfection experiments. The plasmids used were: 1) a reporter construct, pRSVCAT (Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982), in which the CAT gene is controlled by the LTR of Rous sarcoma virus; 2) HIV/2A expressing poliovirus protein 2A; and 3) pSVETA, in which the HIV-1 tat gene is transcribed from an SV40 promoter (Muesing et al., Cell, 48: 691-701 (1987), providing trans-activator function (Figure 3c).

HeLa cells were plated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at about $5x10^5$ per 60mm petri dish 12 to 24 hours prior transfection. The calcium phosphate-DNA precipitates were prepared as described (Sun et al., Genes Devel 2: 743-753 (1988)), and applied to cells without the medium and incubated at 25°C for 15 min before adding fresh medium. Glycerol shock was performed 12 hours after the addition of precipitates. Cells were harvested 48 hours after the addition of precipitates, and CAT activities were assayed as described. The HeLa 2A-38 cell line was established by cotransfecting pHIV/2A with pSV2neo using the calcium phosphate-DNA precipitate method. Transfected cells were selected 48 hours later with 1 mg/ml G418 in DMEM for two weeks. Dozens of colonies were propagated and analyzed by Southern blot for their genomic structures and by cotransfection for the inhibitory function of 2A. It was expected that pHIV/2A would need tat

synthesis from pSVETA for expression of the 2A protein because the HIV LTR is strongly controlled by tat. It was also expected that the expression of 2A would inhibit the translation of pRSVCAT mRNA, and give a very low CAT activity (Figure 3c). Results showed that this was the case: the expression of tat greatly decreased synthesis of CAT when cells were co-transfected with pRSVCAT and pHIV/2A (Figure 3a, compare lanes 3 and 4). Without pHIV/2A, no inhibition was evident (lanes 1 and 2). The single amino acid insertion in 2A greatly impaired the ability of 2A to inhibit mRNA translation (lanes 5 and 6). A deletion in 2A completely abolished its inhibitory function (lanes 7 and 8).

In addition, cotransfection experiments using a pRSVLacZ plasmid (the B-galactosidase gene driven by the LTR of Rous sarcoma virus) (Hall et al., J. Mol. Appl. Genet., 2: 101-109 (1983), or a pSV2CAT plasmid (the CAT gene driven by SV40 early promoter) (Gorman et al., Mol. Cell. Biol., 2: 1044-1051 (1982) as the reporter genes were also carried out. Equivalent results were obtained suggesting that the inhibition of translation by 2A is not specific to a particular protein, promoter or mRNA capping sequence. Thus, it is reasonable to conclude that 2A, even when it is expressed out of the context of poliovirus, inhibits cellular mRNA translation.

The low level of expression from the LTR of HIV-1 without tat expression has made it possible to establish HeLa cell lines permanently carrying pHIV/2A. The plasmid pHIV/2A was cotransfected into

HeLa cells with the plasmid pSV2neo which expressed the neomycin resistance gene. Southern and Berg, J. Mol. Appl. Genet., 1:327-341 (1982). Several dozen individual neomycin resistant colonies were obtained. The expression of the integrated HIV/2A genes could then be activated by transfecting in the tat gene expressed by pSVETA. The inhibitory function of 2A in these cell lines was assayed by cotransfecting pRSVCAT and pSVETA, and measuring the CAT activity of the cotransfected extracts. These cell lines were found to contain variable copies of HIV/2A genes and respond to tat-activation to variable extents. One of the cell lines, 2A-38, carried 10 to 20 copies of HIV/2A gene and translated RSVCAT mRNA more than 10-fold less efficiently when pRSVCAT was cotransfected with pSVETA compared to transfection without pSVETA (Figure 3b).

During poliovirus infection, host cell protein synthesis shut off, and the polypeptide P220, a component of eIF-4F, is cleaved. The poliovirus protein 2A appears to be involved in the later event, both because mutant 2A-1 fails to trigger the cleavage of P220, and because in vitro translated 2A indirectly induces the cleavage of P220 in a HeLa cell extract. Krausslich et al., J. Virol., 61: 2711-2718 (1987). Therefore, experiments were carried out to determine whether 2A, when expressed alone in vivo, could cause the cleavage of P220. The expression of 2A in the cell line 2A-38 was activated by transiently transfecting it with the tat gene and the cleavage products of P220 in the

transfected extract were assayed by an immunoblot using an anti-P220 serum.

Figure 4 demonstrates the cleavage of P220 in 2A-producing HeLa cells. The immunoblot experiment was performed using an antiserum against P220, essentially as described by Bernstein et al., except that an alkaline phosphatase conjugated anti-rabbit IgG (Promega) was used as the second antibody according to the vendor's instructions. Extracts were prepared 48 hours post-transfection from HeLa cells transfected with or without pSVETA (lanes 1 and 2 of Figure 4), and 2A-38 cells transfected with or without pSVETA (lanes 3 and 4 of Figure 4). Extracts were also prepared from HeLa cells infected with wild type (Figure 4, lane 6) or 2A-1 mutant virus (Figure 4, lane 5) at multiplicity of infection of 20 for 3.5 hours. One fifth of the extracts from each 60 mm dish was analyzed by electrophoresis through a 6.5% SDS-polyacrylamide gel. P220 and its cleavage products are indicated in Figure 4.

The cleavage products were apparent in the induced 2A-38 cells (Figure 4, lane 3), but not in the unactivated 2A-38 cells (lane 4) or in normal HeLa cells (lanes 1 and 2). The cleaved products comigrated with those in poliovirus-infected HeLa cells (lane 6). Due to the inefficiency of the calcium phosphate-DNA precipitate transfection method, only a portion of the 2A-38 cells used for transfection would be expected to receive the tat gene, express 2A and induce the cleavage of P220. Thus, in lane 3, the cleavage products were probably

contributed from this portion of the cells, whereas the intact P220 would be from the remaining portion that did not receive the tat gene. Lane 4 also emphasizes that in 2A-38 cells, the baseline expression of 2A protein is so low that no cleavage of P220 is evident.

It was previously evident that a mutation in protein 2A interferes with the virus-induced shutoff of host cell protein synthesis. It was also evident that 2A is a protease that cleaves the P1-2A bond in the poliovirus polyprotein. Because protein 2A is cleaved from precursors, it was not clear that 2A itself was responsible for both effects. The present results show directly that expression of 2A (with a short tail of 2B attached) can cause inhibition of cellular mRNA translation. 2A also acts as a protease because 2A mutants are defective in cleavage of 2A from its precursor. 2A is able to induce cleavage of P220, as was also evident from in vitro results, but the mechanism of cleavage, thought to be an indirect effect of 2A, remains obscure.

Unlike most other retroviruses, the LTR of HIV can be transactivated several hundred-fold by the tat gene from a very low basal level. The level of expression in HeLa cells after activation is much higher than synthesis from RSV LTR or SV40 early promoter. It has been possible to establish 2A-expressing cell lines and, thus, to demonstrate that this feature of the HIV LTR can be utilized as a good activatable system to study the functions of

proteins toxic to cells as well as to design strategies that might counter HIV infection.

EXAMPLE 2 Selective Ablation OF HIV-Infected Cells by Means of a Recombinant Construct

Method: Recombinant constructs which include a minimal HIV LTR promoter and the Herpes simplex virus thymidine kinase (TK) gene were produced and introduced into human cells which are themselves TK$^-$ (i.e., do not contain an endogenous TK gene) and in which the HIV tat gene was present, as described below. The effects on viability of cells containing the tat gene, upon expression of the TK gene, in the presence or absence of acyclovir, were determined.

Constructs containing portions of the HIV promoter, as represented in Figure 5, were produced. Construct A includes the 700 bases of the HIV promoter from the Xho site present at the 5' end through the Hind III site at the 3' end and the Herpes Simplex virus (HSV) thymidine kinase gene; the HSV TK gene includes the region present between the Bgl site (5' end) and the Bam site (3' end). It, thus, includes the HIV NFKB elements, SP-1 sites, the TATA box and the tat site. Construct B includes the same components a construct A except that there are alterations at the sites indicated in the NFKB elements, which result in inactivation of the elements. Constructs A' and B' are the same, respectively, as constructs A and B except that they begin at a Sca site at the 5' end, rather than the 5' Xho site. Constructs C, D and E are shorter

portions of the HIV promoter, as indicated in Figure 5. In each case (Constructs A-E), there are additional intervening sequences present between the regions indicated in Figure 5 which are sequences which normally occur in the HIV promoter.

Each construct has been inserted into at least one retroviral vector, both in direct and in reverse orientation. Constructs produced in this manner have been transfected into Psi Crip cells, which were maintained under conditions appropriate for expression of the plasmid DNA. The viral supernatant from these cells was used to infect human osteosarcoma cells, designated 143 cells, which do not contain the thymidine kinase gene (TK^-). Selection of cells using HAT selection was carried out, resulting in isolation of cells which contained the HIV-TK construct and in which the incorporated TK gene was expressed. The tat gene of HIV was introduced into 143 cells, to produce cells designated 143 tat cells. Subsequent assays were carried out to look for differences in the expression of TK in the two types of cells (143 and 143 tat).

These assessments demonstrated that a proviras integrant was present in all cells (See Figure 6); control cells are those containing control plasmid DNAs. The effects of addition of acyclovir to uninfected 143 cells and 143 tat cells are shown in Figure 7. These controls show that acyclovir has no effect on uninfected control cells. As shown in Figure 7, when 10μM acyclovir was added to cells expressing a recombinant construct (as shown in

Figure 7, 143 tat cells expressing ERH200T or ER165T), all cells were killed. The same was evident at 100um acyclovir. In contrast, no control cells were killed. It should be noted that 143 cells (i.e., those not containing the tat gene) were similarly affected by the two concentrations of acyclovir. This indicates that although the desired effect is evident, it is not as selective (specific to cells expressing tat) as desired and that a construct containing less of the HIV promoter (e.g., construct E of Figure 5) would be more useful. In this construct, the minimal HIV promoter consists of only the TATA element and the tat binding sequence. This minimal promoter has been shown by other laboratories to result in the lowest level of transcriptional activity.

EXAMPLE 3 HIV Infection of H9 Cells Containing a Mutated gag Construct

Method: Immunofluorescence of H9 cells with AIDS patients serum was carried out on days 3 and 4 post infection and the number of cells infected with HIV was determined. Results were also checked by plaque assay of the COS supernatant of a CD4-carrying Hela cell line and patterns observed were comparable.

TABLE 1 Infection of Cos cells with HIV

	Number of cells infected with HIV			
p Rip 7'.HxB2+...[1]	at 3 days		at 4 days	
	#	%	#	%
p918.CAT[2]	164	15	285	60
pRip.An[3]	240	22	279	59
pAmif[4]	220	20	363	76
pAmen[5]	179	16	267	56
gag mutants				
p ΔH10X12.Amen	14	.01	62	13
p ΔH10H17.Amen	54	.05	261	55
p ΔX12H17.Amen	115	10	200	42
p ΔP14A20.Amen	37	.03	93	20
p CH60.Amen[6]	160	15	250	53

Interpretation: The best results were obtained with Δ H10X12 and Δ P14A20 and to a lesser extent, with Δ H10H17.

1 WT HIV
2 pSVCAT
3 WT gag
4 Frame shift mutation in rif
5 WT gag & frameshift in env
6 Linker insertion at approximately position 6000 (in tat-rev)

The same procedure was carried out, using the constructs listed in Table 2. Results obtained on days 4 and 5 post infection are also shown in Table 2. Results are presented as (+) cells/total cells

and % (+) cells. No mock-infected cells were positive. A deposit of the gag mutant designated pΔH10X12 was made (March 3, 1989) in the American Type Culture Collection (Rockville, MD), under terms of the Budapest Treaty, under ATCC #40580.

TABLE 2 Infection of Cos cells with HIV

Rip7'+...	day 4 d4 #/total	%	day 5 d5 #/total	%
2:RipA^n	93/146	63	107/144	75
3:RipAmen	74/147	74	139/196	71
4:CH10	20/174	11.5	75/212	35
5:CP14	3/180	3.5	23/229	10
6:CS15	18/160	11	41/251	16
7:CH17	41/102	40	186/244	76
8:CA20	5/212	2.3	75/228	32
9: H10X12	8/202	3.9	34/286	12
10: H10H17	22/200	11	54/231	22
11: X12H17	54/218	25	151/195	77
12: P14S15	13/200	6.5	132/232	57
13: P14A20	26/180	14.4	98/153	64
14:R7. H10X12	7/210	3.3	52/203	25
15:R7. P14S15	28/200	14	118/203	59
16:R7. H10H17	19/200	9.5	74/196	37

Mock: No positive cells

EXAMPLE 4 Generation of HIV Gag Mutants and a rev⁻ Control

Further efforts which resulted in construction of dominant negative gag mutants and assessment of their effects on HIV were carried out as follows: Initially a number of site-directed lethal mutations in the HIV gag gene was generated. Previous work on murine retroviruses suggested that alterations of a minimum of a few amino acids would be necessary to cause lethality. Lobel, L.I. and S.P. Goff, Proc. Natl. Acad. Sci., USA, 81: 4149-4153 (1984); Schwartzenberg, P., et al, J. Virology, 49:918-924 (1984). Therefore, linker insertional and deletional mutagenesis was performed on a plasmid (W13) carrying a full-length copy of the HIV-HXB2 proviral DNA.

Plasmids and Mutagenesis:

W13 contains the viral insert of HXB2D, Shaw, G.M., et al, Science, 226:1165-1171 (1984) in HXB-Rip7, McCune, J.M., et al, J. Virol., 49:909-917 (1988), with a full nef coding region, as described, Kim, S, et al, cited supra. Mutations were introduced by complete or partial digestion of W13 with restriction enzymes, followed by incubation with the Klenow fragment of DNA polymerase 1 or with T4DNA polymerase, in the presence of nucleoside triphosphates, and in most cases insertion of a Cla 1 linker, before reclosure with T4 DNA ligase. More precisely, the different mutants were obtained as follows. SCH10-W13 (I) and SCH17-W13 (II): partial digestion with Hind III, Klenow, insertion of an

8-mer Cla 1 linker. bCP14-W13 (III) complete digestion with Pst 1, T4 DNA polymerase, 8-mer Cla 1 linker insertion, Klenow; CS15-W13 (IV): complete digestion with Spe 1, Klenow, 8-mer Cla 1 linker insertion; CH60-W13 (V): partial digestion with Hind III, Klenow, 8-mer Cla 1 linker insertion. Two constructs were obtained after deletion of larger fragments, taking advantage of pre-existing or newly introduced restriction sites. ΔH10X12-W13 (VI) had a deletion from the Hind III site located at nucleotide 1084 to the Xmn site at nucleotide 1275, connected through Cla 1 linkers; ΔP14S15-W13 (VII) was obtained by ligating the Cla 1 sites previously introduced at the Pst 1 ((1418) and Spe 1 (1506) sites. WT-ΔE and related constructs were made from W13, by partial digestion with Stu 1 and insertion of an 8-mer Mlu 1 linker at position 6833 (resulting mutation: $AGGCCT_{6836}$ = AGGGACGCGTCCCT), followed by replacement of a Xho 1 - Xba 1 fragment, encompassing the whole 3' LTR, by a Sma 1 - Aat 11 fragment from pBC12/RSV/SEAP, which contains the proinsulin gene 3' polyadenylation signal. Berger, J., et al, Gene, 66:1-10 (1988). R7-dhfr was constructed by insertion of the coding sequence for a mutant dihydrofolate reductase gene which demonstrates a reduced affinity for methotrexate. Simonsen, C.C. and A.D. Levinson, Proc. Natl. Acad. Sci., USA, 80:2495-2499 (1983), between the initiation codon for the nef gene and the proximal broder of the 3' LTR. This virus is replication competent and confers resistance to methotrexate. The R7-Hyg virus was constructed by insertion of the

hygromycin resistance gene, Gritz, L and J. Davies, Gene, 25:179-188 (1983) into the identical region, and is also replication competent. WT-ΔE-dhfr and related constructs were made by replacement of the Xho 1 - Xba 1 fragment in WT-ΔE and others by the corresponding fragment from R7-dhfr. All cloning manipulations followed standard procedures. Maniatis, T., et al, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1982).

Cell Culture, Transfections and Infections:

COS cells were maintained in Dulbecco modified Eagle Medium (DME) supplemented with 5% fetal calf serum and were transfected using DEAE-dextran and chloroquine. Ausubel, F., et al, Current Protocols in Molecular Biology (Wiley, New York) (1987), or calcium-phosphate precipitation, Chen, C. and H. Okayama, Cell Biol., 7:2745-2752 (1987). H9 cells were grown in RPMI supplemented with 10% fetal calf serum. HT4-6C cells, Chesebro, B. and Wehrly, K., J. Virol., Vol. 62: pp 3779-3788 (1988) and derivatives were grown in DME supplemented with 10% fetal calf serum; dhfr-transformed cells were selected and maintained in 2μM amethopterin (Sigma). For the phenotypic analysis of the mutants, COS cells were transfected with 5 μg of plasmid DNA, and the production of viral particles was measured after 60 hours by using a highly sensitive ELISA assay system for p24 gag antigen (DuPont-NEN, Inc., Billerica, MA), following the manufacturer's instructions. An internal control was established by cotransfecting the secreted alkaline phosphatase

(SEAP) gene expression vector pBC12/RSV/SEAP by the method described by Berger, J. et al. and also by checking the percentage of immunofluorescent COS cells, using serum from an HIV-1 seropositive individual as detector antibody. Ho, D.D. et al, Science, 226:451-453 (1984).

A similar procedure was followed to evaluate interference in the transient assay, except that COS cells were co-transfected with W13 and the various ΔE constructs, at a ratio of 1:4. H9 and HT4 cells were infected as described by Kim and co-workers.

Immunoblot Analysis of Proteins:

Cellular proteins were prepared as previously described. Trono, D. et al, J. Virol., 62:2291-2299 (1988). A portion of the cytoplasmic extract was fractionated by electrophoresis through a 15% SDS-polyacrylamide gel, which was then electroblotted onto nitrocellulose. Immunoblot analysis was performed using a Protoblot system (Promega Biotec, Madison, Wis.), following the manufacturer's instructions, with an anti-p24 monoclonal as detector antibody (a gift from F. Veronese).

The nature, location and designation of those mutations are described in Figure 9 and in Tables 3 and 4.

TABLE 3

gag and rev Mutagenesis

construct number	laboratory designation	wild type sequence[a]	mutant sequence[a]
I	SCH10-W13	AGGAAGCT$_{1088}$	AGGAAGCTCATCGATGA
II	SCH17-W13	AGCAAGCT$_{1785}$	AGCAAGCTCATCGATGA
III	bCP14-W13	GAAGCTGCAGAA$_{1422}$	GAAGCCATCGCGATGGAA
		E A A E	E A I A M E
IV	CS15-W13	ACTAGTACC$_{1514}$	ACTAGCATCGATGCTAGTACC
		T S T	T S I D A S T
V	CH60-W13	AAGCTTCTC$_{6034}$	AAGCTCATCGATGAGCTICTC
		K L L	K L I D E L L

[a] Differences between the wild type sequence and the mutant sequence are underlined; numbers in subscript refer to the nucleotide location.

TABLE 4

Plasmid and Cell Lines Used in This Study

	Mutant	Env⁻;poly (A)	+dhfr gene	into HT4 cells
I	gag-terminated	I-ΔE	I-ΔE-dhfr	HT4(I-ΔE-dhfr)
II	gag-terminated	II-ΔE	II-ΔE-dhfr	HT4(II-ΔE-dhfr)
III	p24-insertion	III-ΔE	III-ΔE-dhfr	HT4(III-ΔE-dhfr)
IV	p24-insertion	IV-ΔE	IV-ΔE-dhfr	HT4(IV-ΔE-dhfr)
V	rev-insertion	V-A[a]	V-dhfr[a]	HT4(V-dhfr)[a]
VI	p17/p24-deletion	VI-ΔE	VI-ΔE-dhfr	HT4(VI-ΔE-dhfr)
VII	p24-deletion	VII-ΔE	VII-ΔE-dhfr	HT4(VII-ΔE-dhfr)
	WT	WT-ΔE	WT-ΔE-dhfr	HT4(WT-ΔE-dhfr)

[a] The ΔE mutation was not incorporated into V because its rev phenotype assures that it will not produce ENV protein.

In two constructs (I and II), a stop codon was introduced in the gag gene, resulting in a truncated gag precursor. In two more constructs (III and IV), linker insertion resulted in the change of a few amino acids in the p24 protein. Two other constructs had further modifications creating large internal deletions in the gag precursor, either overlapping the junction between p17 and p24 (VI), or within the p24 protein itself (VII). Because a functional rev has been shown to be necessary for HIV gag production, an additional construct was made to serve as a complete gag-null mutant, by introducing a 4 amino acid change in the N-terminal portion of the Rev protein (V). Feinberg, M.B., et al, Cell, 46:807-817, (1986).

The phenotypic consequences of those mutations were first tested. COS cells were transfected with the different constructs, and the generation of virus particles was scored by measuring the amount of p24 antigen in the supernatant. To assay for the infectivity of the particles that might have been generated, this supernatant was then used to infect H9 cells. As expected, the supernatants of cells transfected with constructs carrying a stop codon in the gag gene (I and II) had no measurable p24 activity, and were non-infectious (Table 5).

TABLE 5

Phenotypic Analysis of Mutants

plasmid[a]	p24 activity in COS supernatant (ng/ml)	immunofluorescent H9 3 weeks after infection
W13 (wild-type)	120	90
I	<0.1	0
II	<0.1	0
III	0.6	0
IV	1	0
V	1	0
VI	<0.1	0
VII	0.6	0

[a] COS cells were transfected with 5 μg of DNA; plasmid pBC12/RSV/SEAP (1 μg) was used as an internal control; at 60 hours post transfection, the supernatant was harvested for measurement of p24 and SEAP activity, and to infect H9 cells. The experiment was repeated three times, and gave consistent results. The level of variability for the internal control "SEAP" was less than 15%.

[b] H9 cells were exposed to equal amounts of COS supernatants, and followed by indirect immunofluorescence, using serum from an HIV-1 seropositive individual as detector antibody.

A similar result was obtained with the construct carrying a deletion of the p17-p24 cleavage site (VI). For these mutants, it would appear that there was no virus released. Cells transfected with constructs III, IV and VII produced p24 activities that were approximately 100-fold lower than wild type, also indicating a major defect in viral assembly and/or release. Furthermore, when H9 cells were exposed to the supernatant obtained from those cells, and subsequently followed by immunofluorescence using serum from an HIV-1 seropositive individual, no positive cells were seen after three weeks, suggesting that the particles generated were non-infectious. Consequently, all of these gag mutations appeared to be lethal. Construct V, the rev mutant, induced p24 activity levels that were less than 1% of wild type; the supernatant of V-transfected cells also appeared to be non-infectious. The linker inserted in this construct, in addition to changing a few amino acids in the N-terminal portion of the rev protein, introduces a stop codon near the 3' end of the tat first exon. However, Northern blot analysis of transfected COS cells showed that V achieved wild type level of the approximately 2 kb mRNA's (not shown). Therefore, the replicative defect in V probably resulted only from its revI mutation.

EXAMPLE 5 HIV Gag Mutants Interfere with the Replication of Wild Type Virus in a Transient Assay

For these experiments, two additional, lethal modifications were introduced in all constructs, including the controls: a translational frameshift in the proximal portion of the env gene, and a replacement of the 3' LTR by the proinsulin gene polyadenylation signal. These alterations had the effect of making the constructs non-transmissible, and also of preventing env expression, so that in later experiments there would be no surface interference. These constructs are designated ΔE (Table 4). The ΔE mutation was not introduced in V, because its rev phenotype assures that it will not produce. Env protein (this construct is therefore designated V-A). COS cells were co-transfected with the intact wild type proviral DNA (W13) and each one of those constructs, at a ratio of 1:4. Forty-eight hours later, the supernatant was used to infect H9 cells. Immunofluorescence assay of the infected H9 cells with serum from an HIV-seropositive individual was used to evaluate the release of infectious particles from the co-transfected COS cells. Co-transfection of the COS cells with W13 and WT-ΔE or V-A gave comparable results: at 4 days post-infection, approximately 70% of the H9 cells were positive by immunofluorescence (Table 6).

TABLE 6

Interference of Mutants with Wild Type HIV Replication in a Transient Assay

construct used to cotransfect COS cells with W13[a]	immunofluorescent H9 at day 4 post infection (%)
WT-ΔE	72
II-ΔE	60
III-ΔE	3.5
IV-ΔE	9
V-A	70
VI-ΔE	2
VII-ΔE	9

[a] COS cells were cotransfected with W13 and the various mutants, at a ratio of 1:4. Supernatants were harvested after 60 hours and used to infect H9 cells. Those were followed by indirect immunofluorescence, using serum from an HIV-1 seropositive individual as detector antibody.

Similarly, II-ΔE, expressing a truncated form of gag, did not interfere significantly. By contrast, co-transfection with W13 and either III-ΔE, IV-ΔE, VI-ΔE or VII-ΔE appeared to reduce dramatically the infectious yield from the COS cells: at 4 days, there was an 8- to 36- fold reduction in the number of H9 cells positive by immunofluorescence (2 to 9%). Thus, these gag variants had a major inhibitory effect on the generation of infectious particles from the wild type provirus; they represent dominant negative HIV mutants.

EXAMPLE 6 Cells Constitutively Expressing Gag Variants Show an Impaired Ability to Support HIV Replication

To confirm the results obtained from the transient experiments, cells which constitutively express those HIV gag variants were created and assayed for their ability to support HIV replication. A cell line, designated HT4(VI-ΔE-dhfr) which constitutively expresses a gag mutant has been produced.

A modified version of the full-length HIV proviral DNA was used for expression. The previously mentioned 5' env translational frameshift was incorporated to prevent CD4 blockade by the envelope proteins, preserving the infectibility of the transfected cells. To select for cells that had incorporated the constructs, the nef reading frame was replaced by the mutant dihydrofolate-reductase gene (dhfr), which confers resistance to methotrexate. Simonsen, C.C. and Levinson, A.D., Proc.

Natl. Acad. Sci. USA, 80:2495-2499 (1983). Mutants, except V, were transferred into this backbone, and the resulting constructs are designated as ΔE-dhfr (Figure 10 and Table 4). V was transferred to a similar backbone, without the ΔE mutation. HT 4-6C cells (a HeLa cell line expressing the CD4 molecule at its surface, a gift from B. Chesebro) were transfected with those plasmids and placed under methotrexate selection. Grossly similar numbers of resistant colonies were obtained in all cases. Clones were picked, and the presence of the viral DNA was checked by polymerase chain reaction, using primers hybridizing to the HIV LTR.

The cells were further analyzed by immunofluorescence, using serum from an HIV-1 seropositive individual. As predicted, cells transformed with I-ΔE-dhfr and V-dhfr showed no immunofluorescence, whereas cells transformed with the other constructs were positive (not shown). Western blot analysis of cellular proteins using the same antiserum confirmed that none of those clones expressed the Env protein (not shown). Western blot analysis was also performed using an anti-p24 monoclonal antibody (a gift from F. Veronese) (Figure 10). No gag protein was detected in HT4(I-ΔE-dhfr)and HT4(V-dhfr) (not shown); HT4(WT-ΔE-dhfr) (lane 3) and HT4(III-ΔE-dhfr) (lane 4) showed a normal pattern of gag protein, compared to cells infected with a replication competent version of HIV-1, HT4(R7-dhfr) (lane 2); finally, HT4(VI-ΔE-dhfr) (lane 5) exhibited a shorter version of the $Pr55^{gag}$ precursor, and no evidence of cleaved

p24 protein, in agreement with the removal of the p17/p24 cleavage site. As a further analysis, p24 activity was measured in the supernatant of those cells (Table 7).

TABLE 7

p24 Activity in the Supernatant of HT4 Cell Lines (ng/ml)

Cell Line	p24 (ng/ml)
HT4(WT-ΔE-dhfr)[a]	350
HT4(I-ΔE-dhfr)	<0.1
HT4(III-ΔE-dhfr)	30
HT4(V-dhfr)	1
HT4(VI-ΔE-dhfr)[b]	<0.1

[a] p24 activity in cytoplasm: 1800; ratio cyt./sup.: 5.1:1

[b] p24 activity in cytoplasm: 625; ratio cyt./sup.: >6250:1

No activity was detected in the supernatant of HT4(I-ΔE-dhfr); HT4(V-dhfr) and HT4(III-ΔE-dhfr) gave levels three-hundred-fold and twelve-fold lower than HT4(WT-ΔE-dhfr), respectively; in the supernatant from HT4(VI-ΔE-dhfr), as predicted from the result obtained during transfection (Table 5), no p24 activity was detected (Table 7). In addition, a comparison of p24 activity in the cytoplasm and the supernatant of HT4(WT-ΔE-dhfr) and HT4(VI-ΔE-dhfr) was carried out. Results are presented in Table 7. This confirmed that the absence of reactivity of the supernatant from VI did not come from an inability of the antiserum to recognize the truncated polypeptide, but rather from a major defect in viral release (ratio cytoplasm/supernatant >6250, in contrast to 5 for WT); this defect was also confirmed by measuring reverse transcriptase activity in the supernatant (not shown). Finally, HT4(I-ΔE-dhfr) and HT4(V-dhfr) were found to fully transactivate a HIV LTR-CAT construct, reflecting high levels of tat expression (not shown).

The ability of the transformed cells to support HIV replication was then tested. For this, HT4(ΔE-dhfr) cells were plated at low density, and exposed to 1 ml. of supernatant from acutely infected H9 cells, or from uninfected H9 (mock-infection). Twelve hours later, the cells were rinsed and placed in fresh medium, and monitored by measuring daily p24 activity in the medium. The result is shown in Figure 12, with values obtained for mock-infected cells subtracted

in each case. Release of p24-containing particles was highest from HT4(WT-ΔE-dhfr) and HT4(V-dhfr), while it was significantly lower from HT4(VI-E-dhfr) cells, suggesting interference with virion assembly and/or release in these cells. HT4(I-ΔE-dhfr) and HT4(III-ΔE-dhfr) cells gave intermediate values. In order to determine whether the particles released in each case were infectious, the supernatants from these five cell lines at day four post-HIV infection were used to infect fresh H9 cells. H9 cells were inoculated with equal amounts of supernatant from the HIV-infected HT4(ΔE-dhfr) cells, harvested at day 4 post infection. After adsorption, cells were washed, placed in fresh medium, and infection was monitored by measuring p24 activity in the culture medium. H9 cells initially exposed to supernatants from HT4(WT-ΔE-dhfr), HT4(I-ΔE-dhfr) and HT4(V-dhfr) showed rapidly increasing values of p24 activity in the culture medium, reflecting initial innoculation by highly infectious virus (Figure 13). By contrast, dramatically slower kinetics of infection were observed in the case of H9 cells innoculated with the supernatant from HT4(III-ΔE-dhfr) and HT4(VI-ΔE-dhfr). This confirmed that very few infectious particles had been originally released from those cells. A direct comparison of two supernatants which contained grossly comparable amounts of p24 activity (see Figure 12) is revealing: the supernatant from HT4(I-ΔE-dhfr) is highly infectious, whereas the supernatant from HT4(III-ΔE-dhfr) appears to contain mostly non-infectious particles (see Figure 13). The exact

amount of interference exerted by III and VI on wild type virus growth cannot be ascertained from these experiments, because of the intrinsically semi-quantitative nature of the assay.

Lastly, it was also demonstrated that the impaired ability to support HIV replication in HT4(III-ΔE-dhfr) and HT4(VI-ΔE-dhfr) was not due to the loss of the CD4 receptor during the cloning procedure. It was also confirmed that the block generated by the gag variants affected the late stages of the virus life cycle. For this, the experiment was first repeated, not in cloned cell lines, but in pooled populations; results comparable to those shown in Figures 12 and 13 were obtained. The different cell lines were then infected with a replication competent variant of HIV-1 virus, R7-Hyg, in which the nef open reading frame has been replaced by the hygromycin resistance gene. This virus confers hygromycin resistance to the cells it infects. Comparable numbers of hygromycin resistant colonies were obtained in all cases. Because the expression of the hygromycin resistance gene was directed by the viral LTR, the finding of resistant colonies depended on the efficient accomplishment of all early steps of the HIV life cycle: entry, uncoating, reverse transcription, integration and gene expression. Therefore, it was concluded that the dominant negative effect exhibited by the gag mutations in III and VI is exerted at a late step of the viral life cycle, concomitant with or subsequent to viral assembly.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 28, line 20 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
01 MARCH 1989 (01.03.89)	40578

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European Patent is sought, the Applicant hereby informs the European Patent Office under European Rule 28(4) that, until the publication of the mention of the grant of the European Patent or until the date on which the European Application has been refused or is withdrawn or is deemed to be withdrawn, the availability of the biological material deposited with the American Type Culture Collection under Accession No. 40578 shall be effected only by the issue of a sample to an expert nominated by the requester in accordance with European Rule 28(5).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Nathaniel R. Hayden
PCT INTERNATIONAL SERVICES DIVISION
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau was

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Form PCT/RO/134 (January 1981)

(January 1985)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 41, line 3 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

American Type Culture Collection

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [5]	Accession Number [6]
03 MARCH 1989 (03.03.89)	40580

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European Patent is sought, the Applicant hereby informs the European Patent Office under European Rule 28(4) that, until the publication of the mention of the grant of the European Patent or until the date on which the European Application has been refused or is withdrawn or is deemed to be withdrawn, the availability of the biological material deposited with the American Type Culture Collection under Accession No. 40580 shall be effected only by the issue of a sample to an expert nominated by the requester in accordance with European Rule 28(5).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

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E. ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

Nathaniel R. Hayden
PCT INTERNATIONAL SERVICES DIVISION
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1985)

(January 1985)

CLAIMS

1. A recombinant construct comprising a) all or a portion of the HIV LTR, or functional equivalent thereof and b) DNA of non-HIV origin selected from the group consisting of DNA which encodes a product toxic to HIV-infected cells and mutated HIV DNA encoding a product which inhibits HIV replication in mammalian cells, under conditions appropriate for expression of said recombinant construct, for use in a method of inhibiting HIV and HIV-infected mammalian cells comprising introducing said construct into said cells.

2. A construct according to Claim 1 wherein the DNA of non-HIV origin encodes a product toxic to HIV-infected mammalian cells or a product toxic to HIV-infected mammalian cells in the presence of a selected substance.

3. A construct according to Claim 1 wherein the mutated HIV DNA encodes a mutated product provided in trans.

4. A recombinant construct comprising all or a portion of the HIV LTR and non-HIV DNA encoding a product toxic to HIV-infected mammalian cells, expression of said non-HIV DNA being under the control of the HIV LTR and occurring only in HIV-infected mammalian cells, for use in a method of selective ablation of

HIV-infected mammalian cells comprising introducing said construct into said cells.

5. An HIV gag mutant for use in a method of inhibiting replication of HIV in a mammalian cell comprising conferring on the cell a dominant negative phenotype by introducing said gag mutant into said cells, either directly or through a precursor of said cell.

6. An HIV gag mutant according to Claim 5 which is selected from the group consisting of:

a) a gag mutant containing an internal deletion which overlaps the p17-p24 cleavage site present in wild type HIV;

b) a gag mutant which encodes at least one amino acid not encoded by the corresponding gag region of wild type HIV; and

c) a gag mutant containing a large internal deletion.

7. An HIV gag mutant according to Claim 6 wherein the nucleotide sequence of the gag mutant of (b) includes the nucleotide sequence GAAGCCATCGCGATGGAA and the nucleotide sequence of the corresponding region of the wild type HIV gag region is GAAGCTGCAGAA, wherein the terminal A of the wild type HIV is at nucleotide position 1422.

8. An HIV gag mutant according to Claim 6 wherein the nucleotide sequence of the gag mutant of (b) includes the nucleotide sequence ACTAGCATCGATGCTAGTACC and the nucleotide sequence of the corresponding region of the wild type HIV gag region is GAAGCTGCAGAA.

9. A recombinant construct comprising all or a portion of the HIV LTR and a mutated portion of the HIV genome, the mutated portion encoding a mutated HIV product provided in trans and capable of inhibiting the corresponding HIV gene product in HIV-infected mammalian cells, for use in a method of inhibiting HIV replication in HIV-infected mammalian cells comprising introducing said construct into said cells.

10. The construct according to Claim 9 wherein the encoded mutated HIV protein is mutated gag protein.

11. A recombinant construct comprising all or a portion of the HIV LTR, or a functional equivalent thereof, and DNA of non-HIV origin encoding a product toxic to HIV-infected mammalian cells, for use in a method of inhibiting HIV function in mammalian cells infected with HIV comprising introducing said construct into said cells, under conditions appropriate for expression of the recombinant construct.

12. A construct according to Claim 11 wherein the DNA of non-HIV origin encodes a product whose expression in the HIV-infected cells is under the control of the HIV LTR and whose expression results in inhibition of HIV in said cells.

13. A recombinant construct comprising all or a portion of the HIV LTR and DNA encoding poliovirus protein 2A, the DNA encoding poliovirus protein 2A being located in the construct in such a manner that its expression is under the control of the HIV LTR, for use in a method of inhibiting HIV function in mammalian cells infected with HIV comprising introducing said construct into said cells.

14. A recombinant construct comprising a) all or a portion of the HIV LTR, or functional equivalent thereof, and b) DNA of non-HIV origin which encodes a product toxic to mammalian cells infected with HIV when present in said cells in the presence of a selected substance, for use in a method of inhibiting HIV in mammalian cells infected with HIV, comprising introducing said construct into said cells in the presence of said selected substance under conditions appropriate for expression of said recombinant construct in said mammalian cells.

15. A construct according to Claim 14 wherein the portion of the HIV LTR is all or a portion of

the HIV promoter, the DNA of non-viral origin is the Herpes simplex virus thymidine kinase gene and the selected substance is acyclovir or an analogue thereof.

16. A recombinant construct, capable of expression in mammalian cells, selected from the group consisting of a) the HIV LTR or a portion of the HIV LTR which includes a functional HIV promoter and DNA of non-HIV origin encoding a product which is toxic to HIV-infected cells, when present in such cells alone or in conjunction with a selected substance, and b) the HIV LTR or a portion of the HIV LTR which includes a functional HIV promoter and mutated or altered HIV DNA.

17. A recombinant construct, capable of expression in mammalian cells, comprising all or a portion of the HIV LTR and non-HIV DNA encoding a product whose expression in HIV-infected cells results in inhibition of HIV in said cells, expression of said non-HIV DNA being under the control of the HIV LTR.

18. A recombinant construct of Claim 17 comprising all or a portion of the HIV promoter and DNA encoding poliovirus protein 2A.

19. A recombinant construct capable of expression in mammalian cells, comprising all or a portion of a) the HIV promoter, and b) DNA of non-HIV

origin which encodes a product which is toxic to HIV-infected mammalian cells, but is not toxic to mammalian cells not infected with HIV, when present in mammalian cells in conjunction with a selected substance, expression of the DNA of non-HIV origin being under control of the HIV promoter.

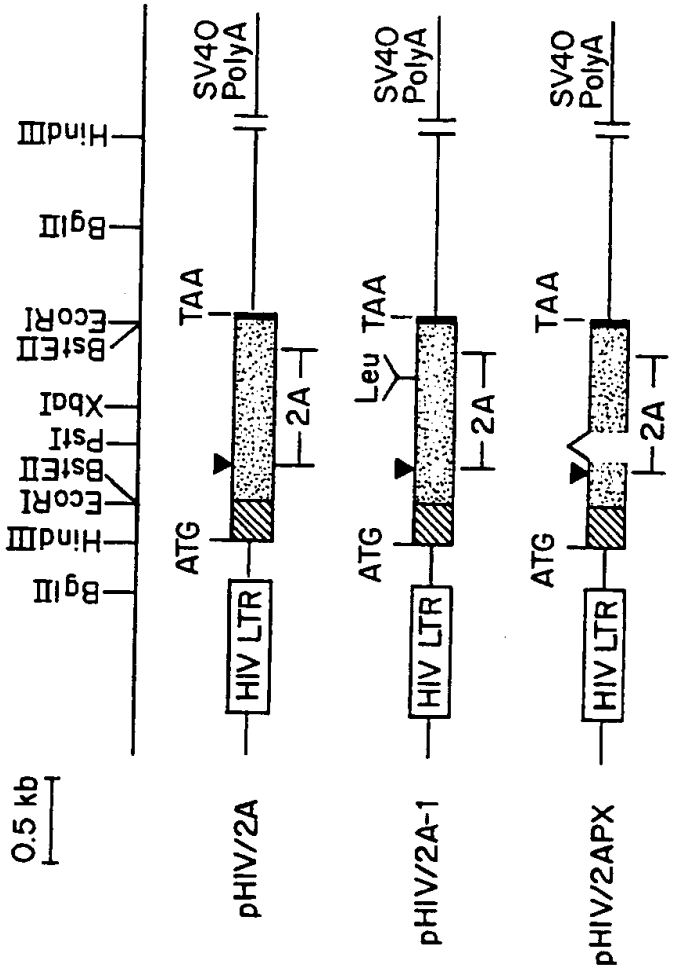
20. A recombinant construct of Claim 19 comprising all or a portion of the HIV promoter and all or a portion of the Herpes simplex virus thymidine kinase gene.

21. A pharmaceutical composition for administration to a human for the purpose of inhibiting HIV present in cells of said human, comprising a recombinant construct capable of expression in human cells, said recombinant construct comprising a) all or a portion of the HIV LTR, which includes a functional HIV promoter, and b) DNA of non-HIV origin encoding a product toxic to HIV-infected mammalian cells.

22. A pharmaceutical composition for administration to a human for the purpose of inhibiting HIV present in cells of said human, comprising a recombinant construct capable of expression in human cells, said recombinant construct comprising a) all or a portion of the HIV promoter and b) a mutated portion of the HIV genome, said mutated portion encoding a mutated HIV product provided in trans and capable of

inhibiting the corresponding HIV gene product in HIV-infected human cells.

1/13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EcoRI
BstEII
XbaI
PstI
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EcoRI
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SV40 PolyA
TAA
2A
ATG
HIV LTR
pHIV/2A
SV40 PolyA
Leu TAA
2A
ATG
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2A
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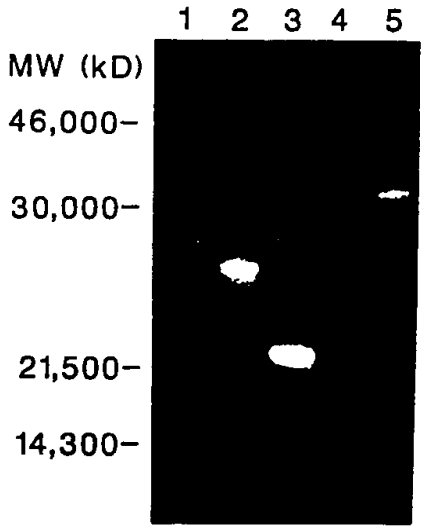
HIV LTR
pHIV/2APX

Fig. 1

FIG.2a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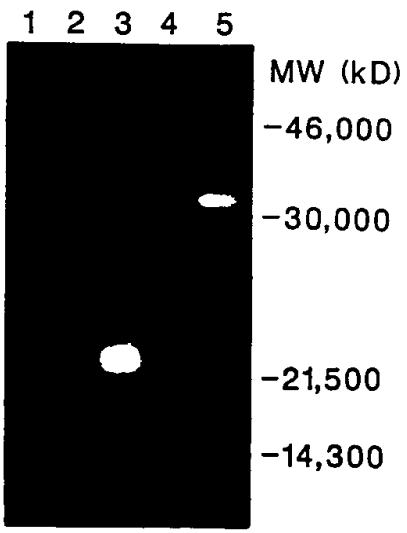
30,000-
21,500-
14,300-

FIG.2b


1 2 3 4 5
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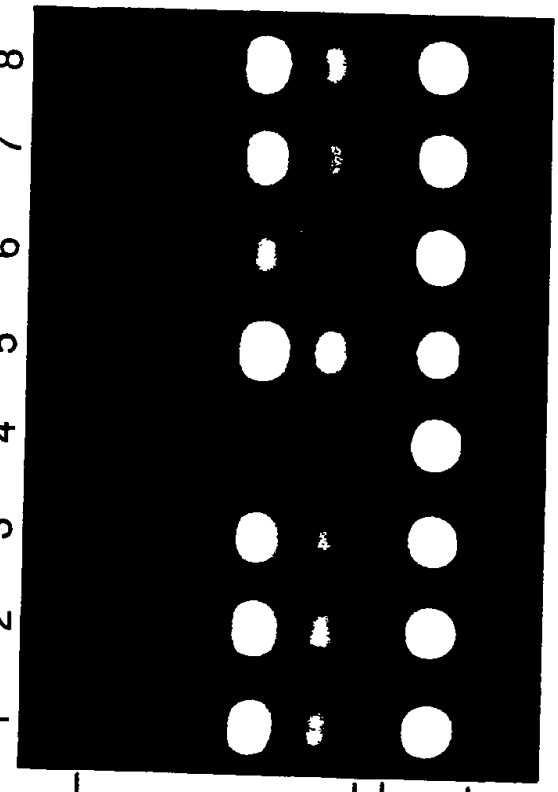
-14,300

FIG.3a


1 2 3 4 5 6 7 8
AC
CM
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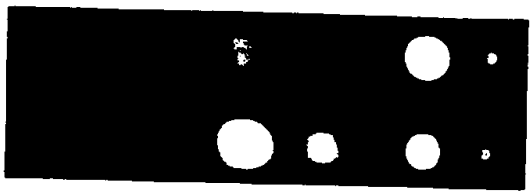
Percent of
Conversion 39.2 42.2 29.6 0.6 71.1 12.4 34.4 42.7

FIG.3b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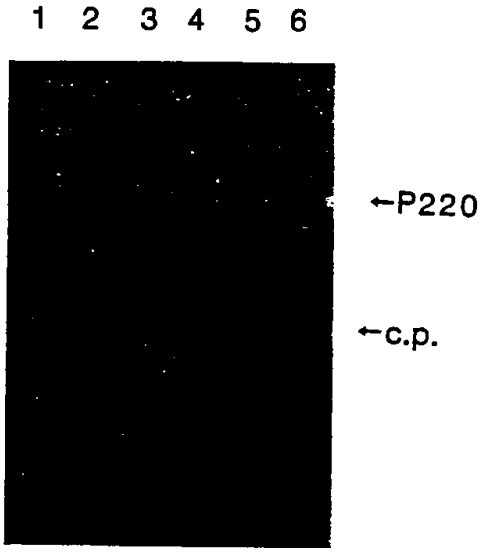
2A-38
— +
82.2 7.3

4/13

FIG. 4



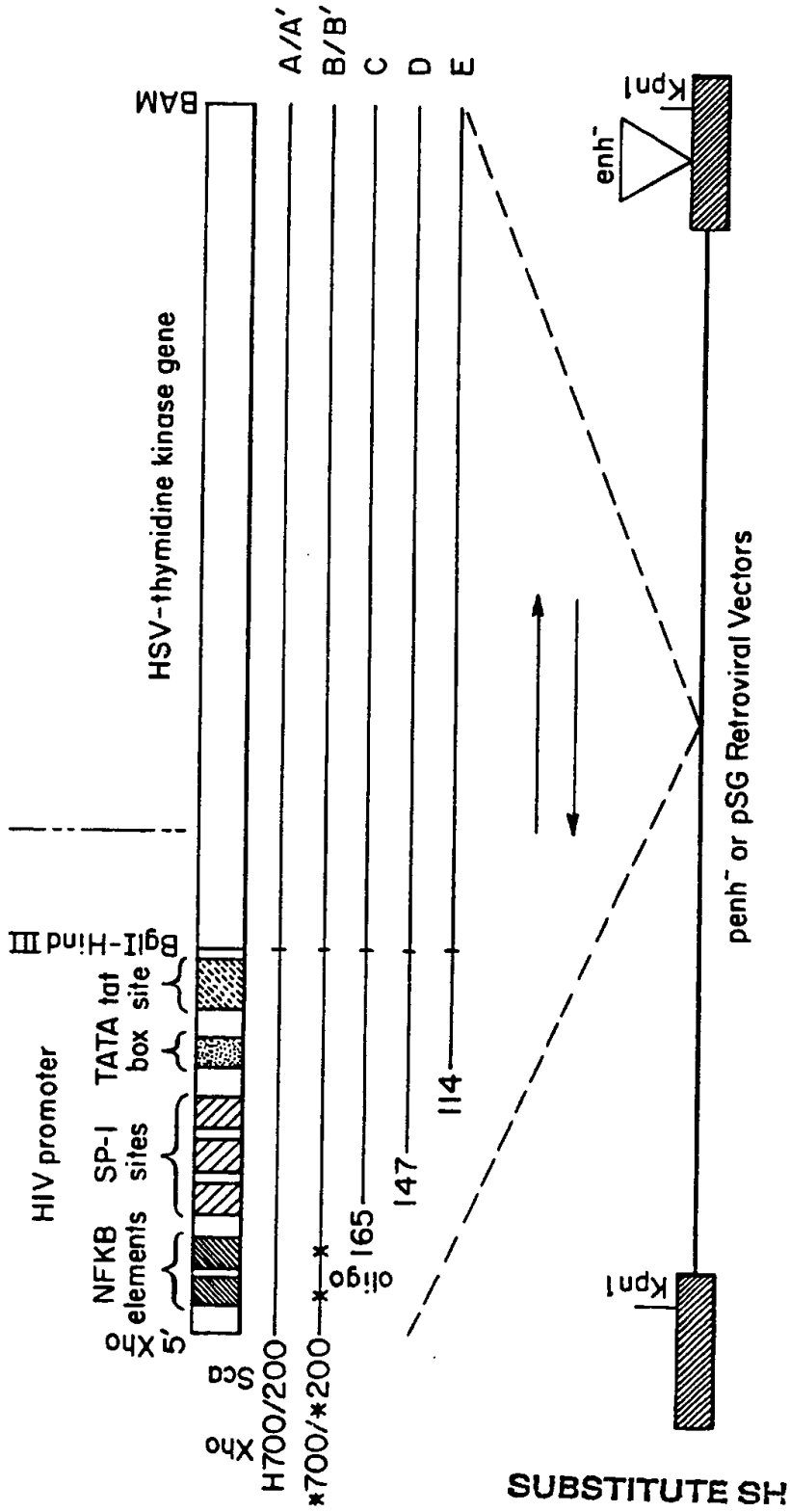

A/A'
B/B'
C
D
E
BAM
HSV-thymidine kinase gene
Kpn1
enh⁻
penh⁻ or pSG Retroviral Vectors
Kpn1
BglI-HindIII
HIV promoter
TATA box
tat site
SP-I sites
NFKB elements
5' Xho
Sca
Xho
H700/200
*700/*200
oligo
165
147
114

Fig. 5

FIG. 6

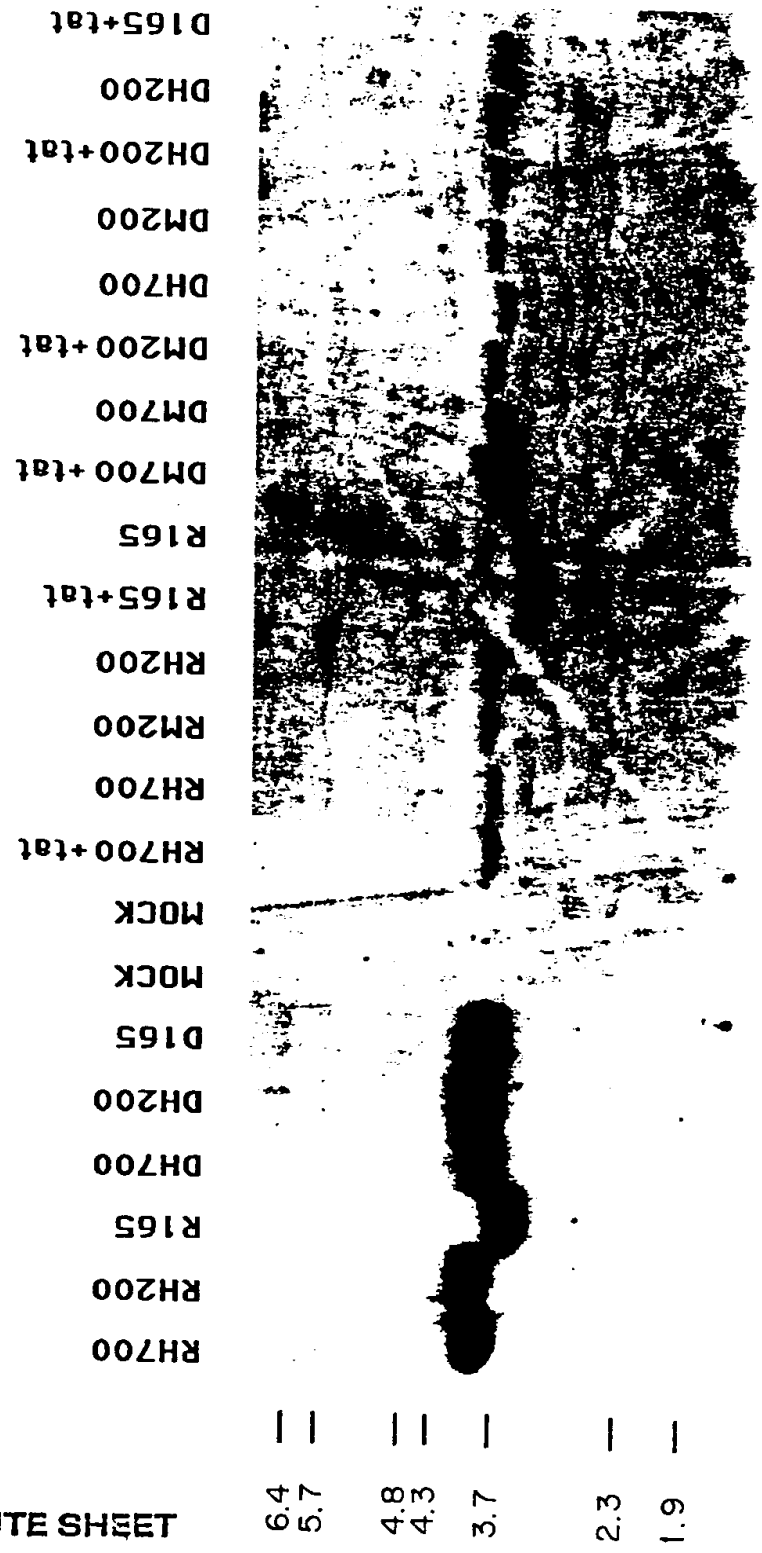
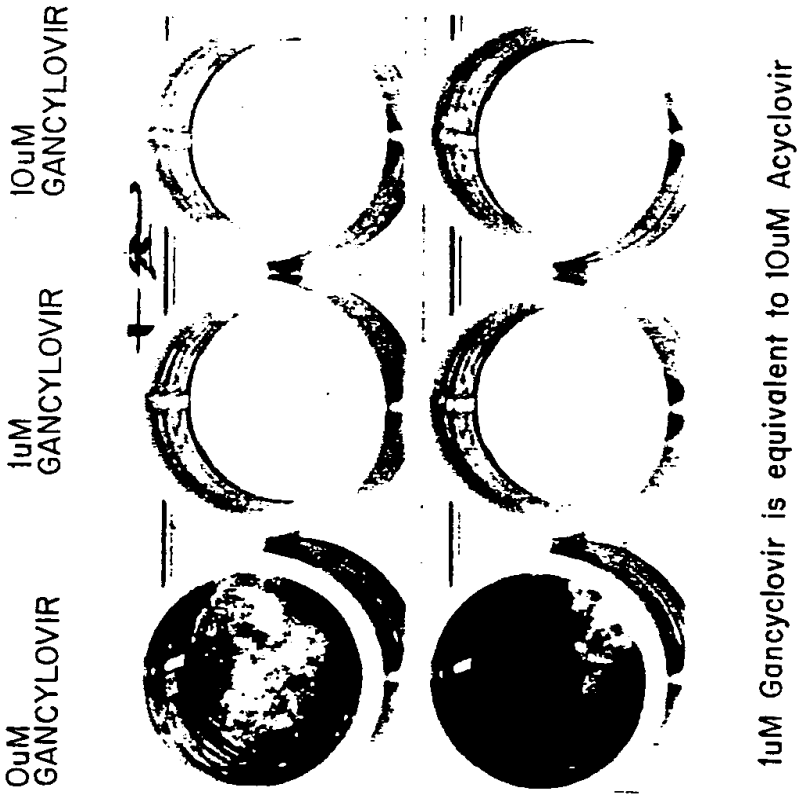

RH700
RH200
R165
DH700
DH200
D165
MOCK
MOCK
RH700+tat
RH700
RH200
RM200
R165+tat
R165
DM700+tat
DM700
DM200+tat
DH700
DM200
DH200+tat
DH200
D165+tat
6.4
5.7
4.8
4.3
3.7
2.3
1.9
CONTROLS
Infected 143 or 143 tat Cells

FIG.7


0uM GANCYLOVIR
1uM GANCYLOVIR
10uM GANCYLOVIR
143-tat
143
1uM Gancyclovir is equivalent to 10uM Acyclovir

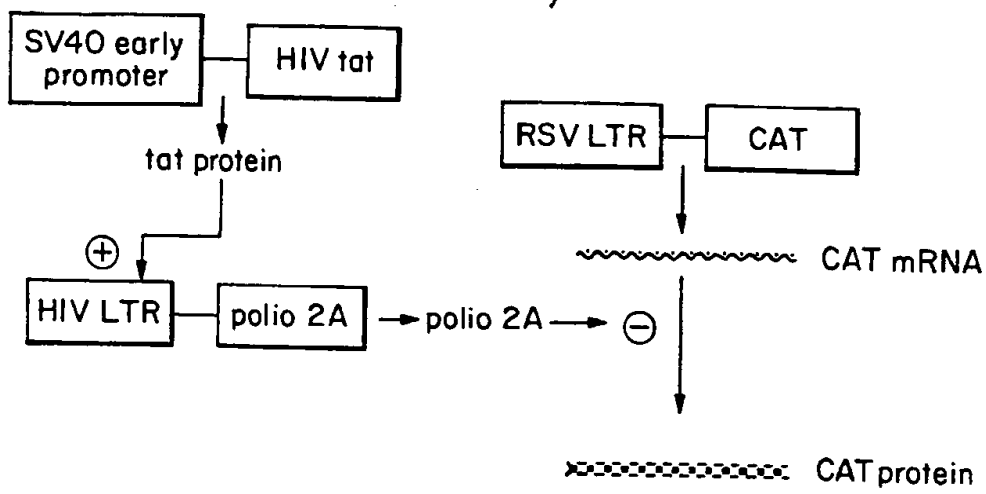

SV40 early promoter
HIV tat
tat protein
RSV LTR
CAT
CAT mRNA
HIV LTR
polio 2A
polio 2A
CAT protein

Fig. 3c

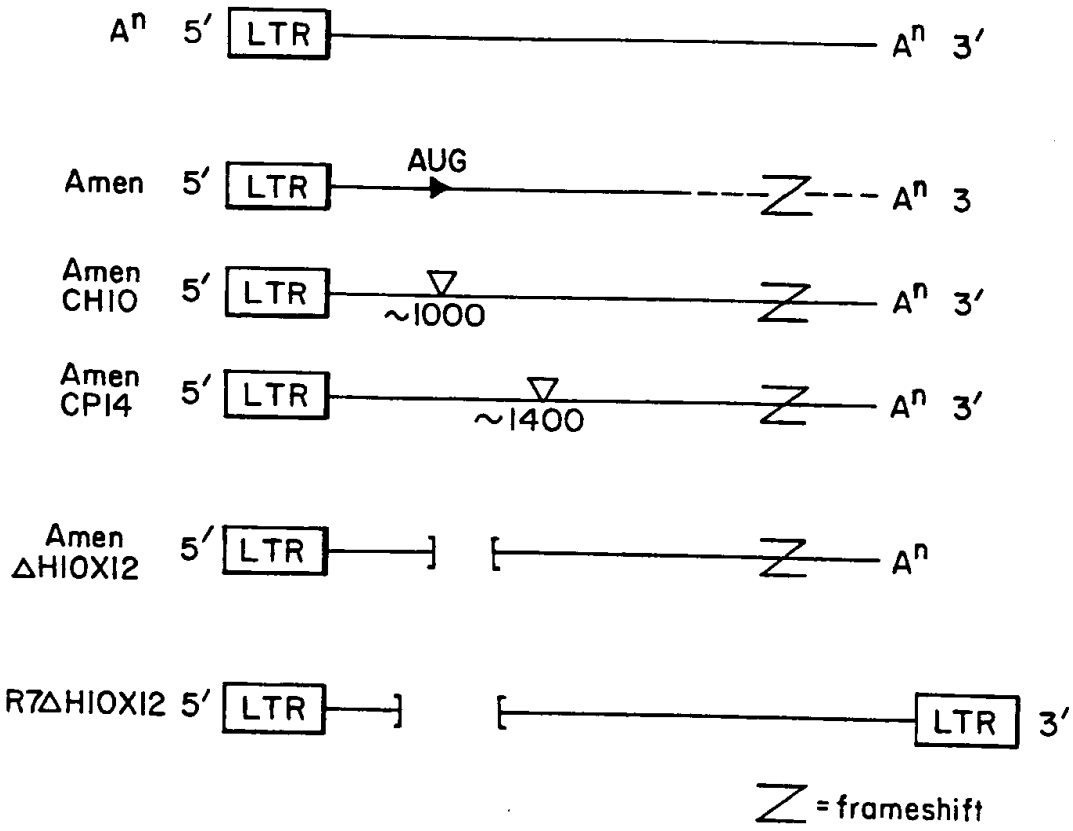

An 5' LTR An 3'
Amen 5' LTR AUG An 3
Amen CH10 5' LTR ~1000 An 3'
Amen CP14 5' LTR ~1400 An 3'
Amen ΔH10X12 5' LTR An
R7ΔH10X12 5' LTR LTR 3'
= frameshift

Fig. 8

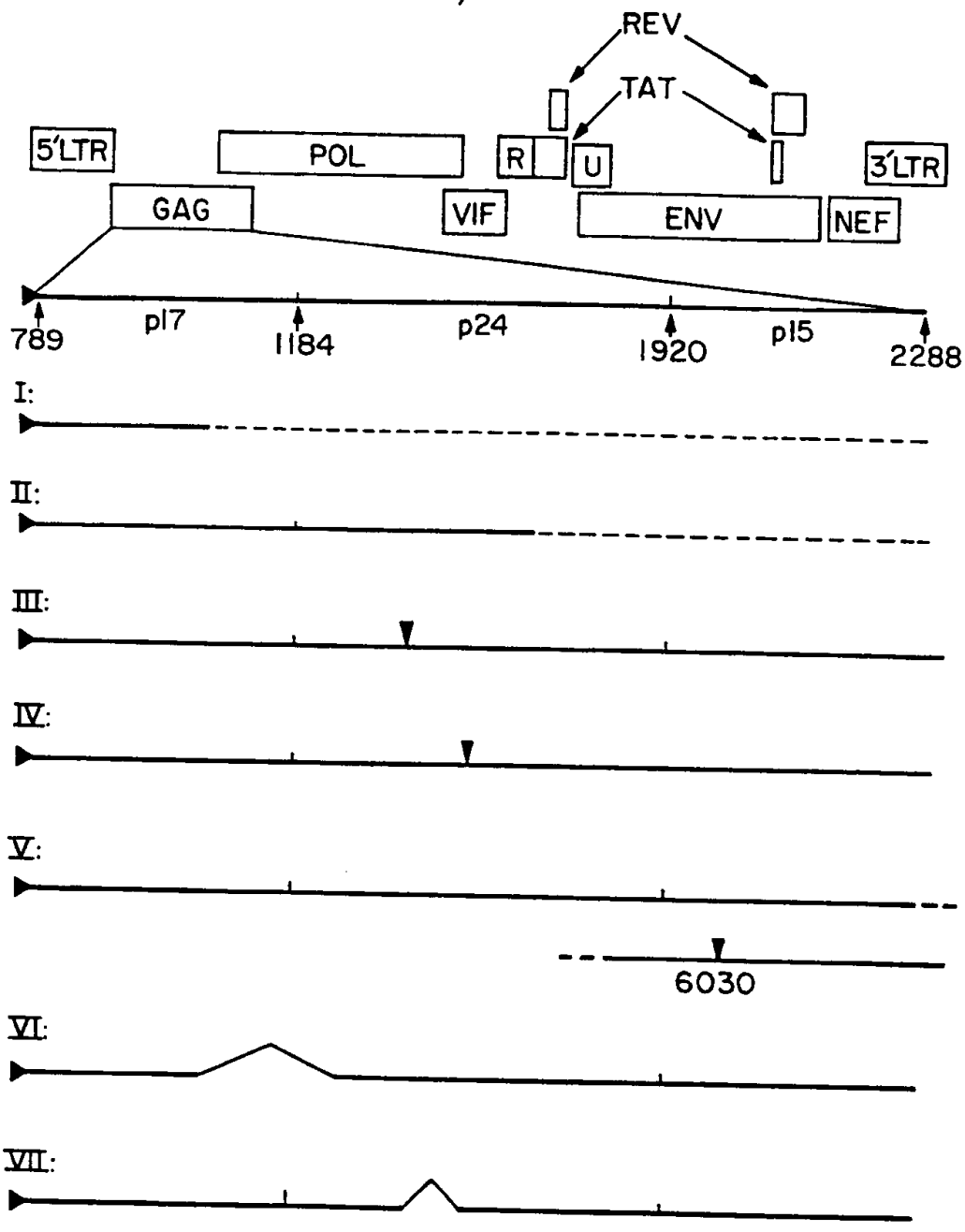

REV
TAT
5'LTR
POL
R
U
3'LTR
GAG
VIF
ENV
NEF
789
p17
1184
p24
1920
p15
2288
I:
II:
III:
IV:
V:
6030
VI:
VII:

Fig. 9

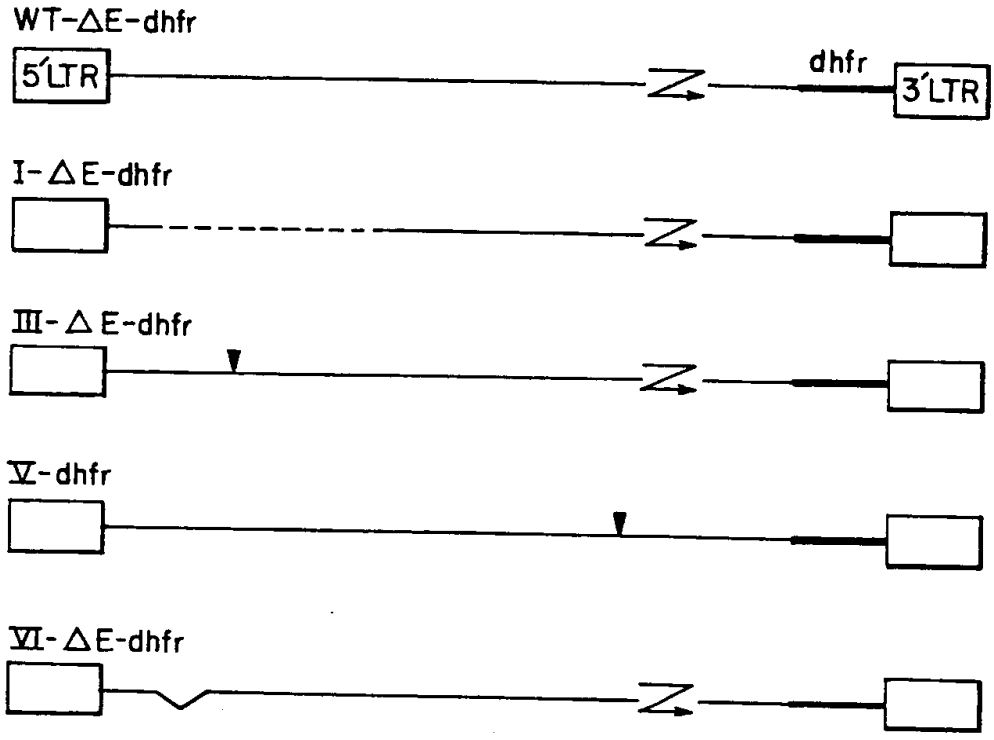
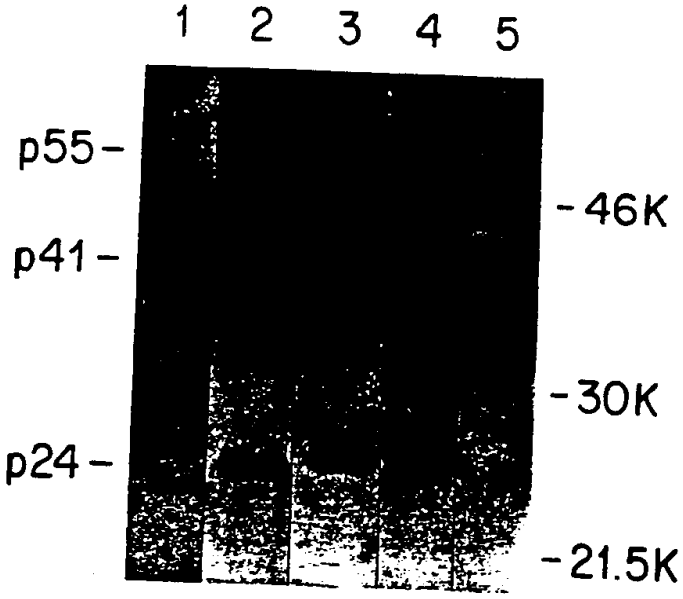


Fig. 10

FIG.11



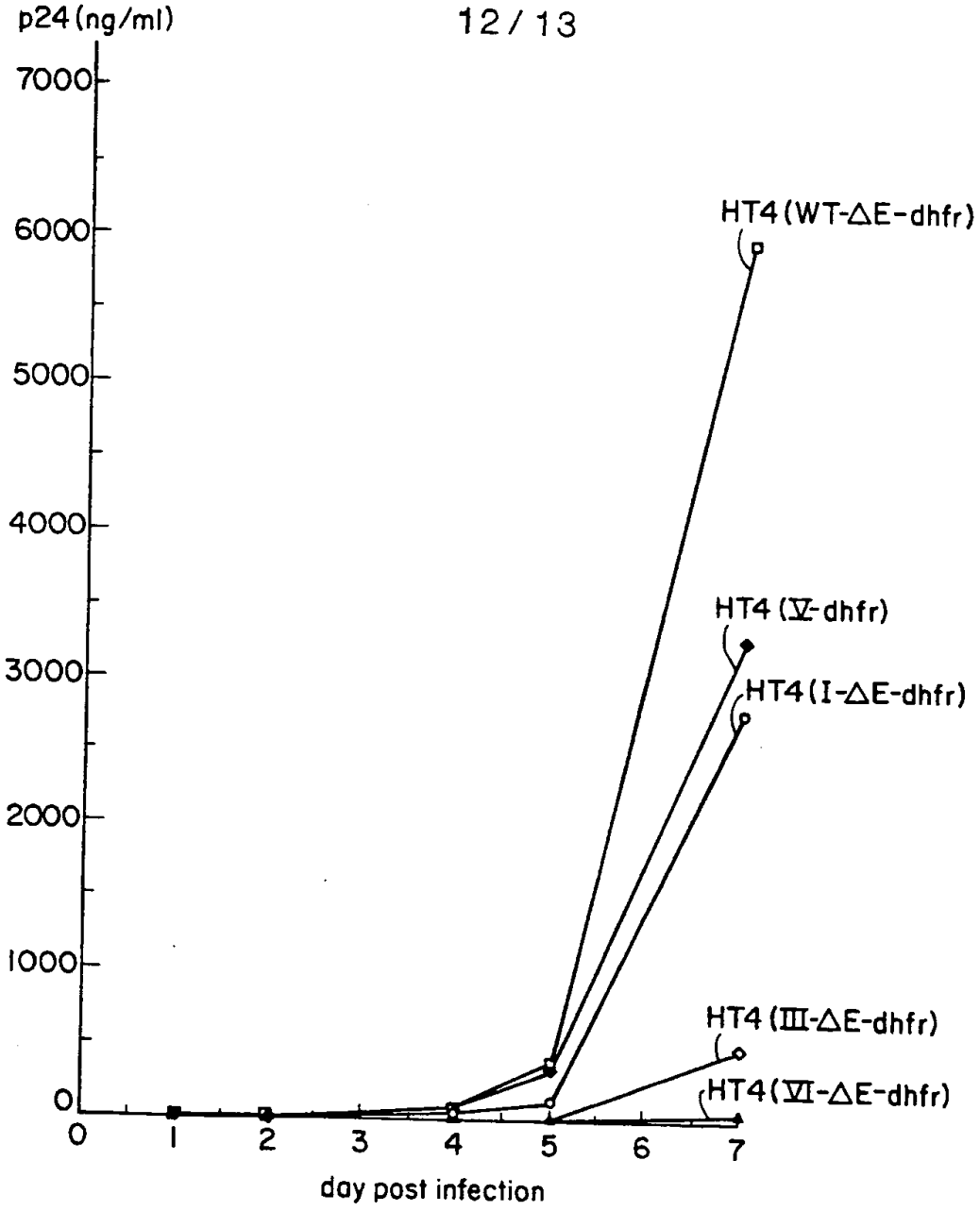

p24 (ng/ml)
7000
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
day post infection
HT4 (WT-ΔE-dhfr)
HT4 (V-dhfr)
HT4 (I-ΔE-dhfr)
HT4 (III-ΔE-dhfr)
HT4 (VI-ΔE-dhfr)

Fig. 12

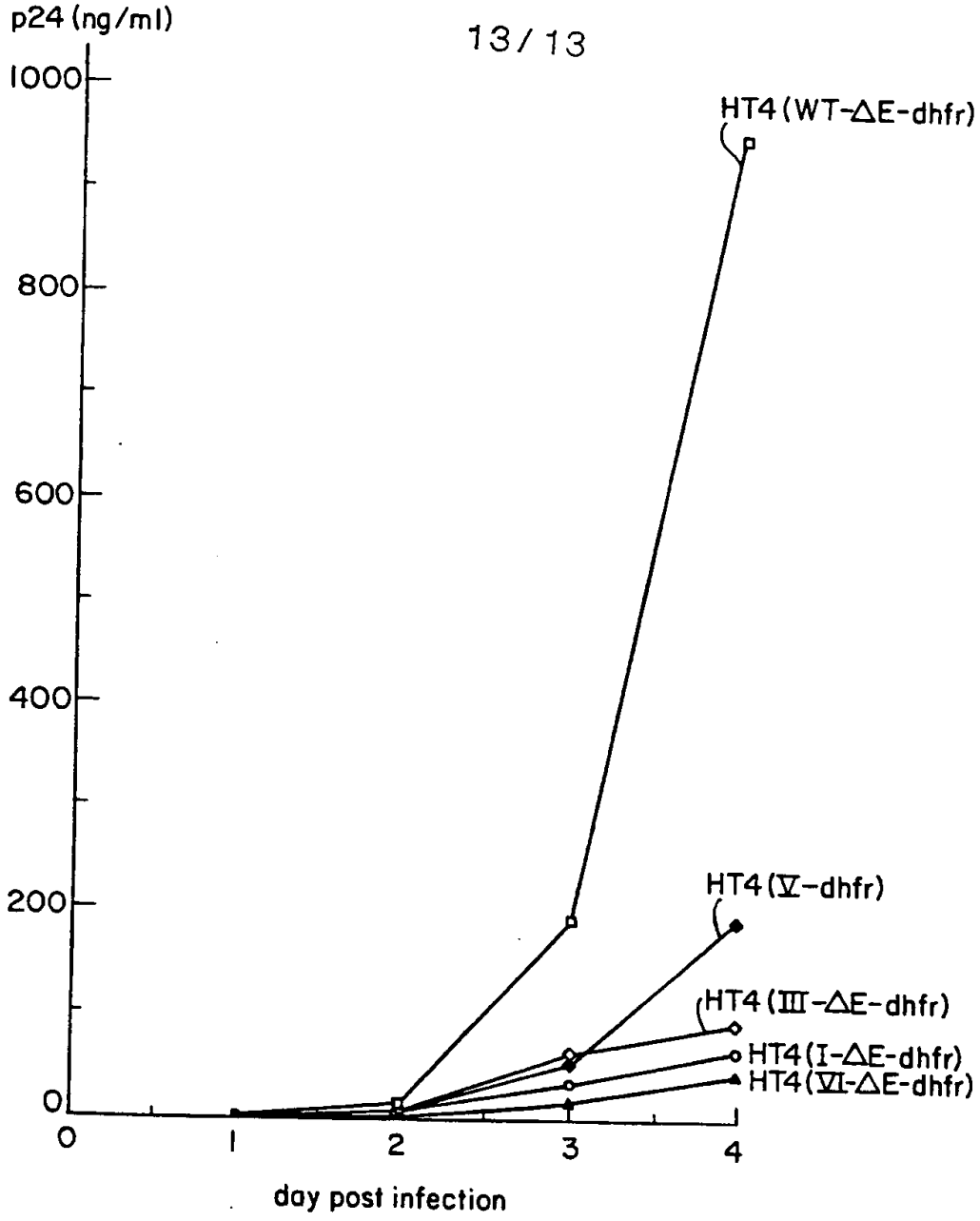

p24 (ng/ml)
1000
800
600
400
200
0
0
1
2
3
4
day post infection
HT4 (WT-ΔE-dhfr)
HT4 (Ⅴ-dhfr)
HT4 (Ⅲ-ΔE-dhfr)
HT4 (I-ΔE-dhfr)
HT4 (Ⅵ-ΔE-dhfr)

Fig. 13

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/01266

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/49, C 12 N 7/00, C 12 N 15/43, C 12 N 15/38, A 61 K 39/21, //A 61 K 48/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Nature, vol. 335, 29 September 1988, A.D. Friedman et al.: "Expression of a truncated viral trans-activator selectively impedes lytic infection by its cognate virus", pages 452-454 see page 454, right-hand column, paragraph 1 --	1,3-10,21,22
A	Nature, vol. 335, 29 September 1988, D. Baltimore: "Intracellular immunization", pages 395-396 see pages 395-396 cited in the application --	1,3-10,21,22
P,X	Cell, vol. 59, no. 1, October 1989, (Cambridge, MA, US), D. Trono et al.: "HIV-1 gag mutants can dominantly interfere with the replication of the Wild-type virus", pages 113-120 see the whole article --	1,3-10,21,22

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th July 1990	06 SEP 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Cell, vol. 58, no. 1, 14 July 1989, (Cambridge, MA, US), M.H. Malim et al.: "Functional dissection of the HIV-1 rev trans-activator - Derivation of a trans-dominant repressor of rev function", pages 205-214 see the whole article; especially page 212 --	1,3,4,9,21, 22
P,X	Cell, vol. 58, no. 1, 14 July 1989, (Cambridge, MA, US), M. Green et al.: "Mutational analysis of HIV-1 tat minimal domain peptides: Identification of trans-dominant mutants that suppress HIV-LTR-driven gene expression", pages 215-223 see page 222, column 1, paragraph 2 --	1,3,4,9,21, 22
P,A	EP, A, 0331939 (GREATBATCH GEN-AID, LTD) 13 September 1989 see page 18, example 17 ----------	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9001266 SA 36602

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 31/08/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0331939	13-09-89	None	

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82